Natural Killer Cell Homeostasis is Under the Control of Transforming Growth Factor-β

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April 1, 2012

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ABSTRACT

Natural killer (NK) cells are members of the innate immunity that respond rapidly to provide the first line of defense against pathogen invasion and cancer. NK cells are produced in the bone marrow and home to the spleen, blood, liver, and lymph nodes where they exist in small numbers that are maintained by homeostasis. Few soluble factors have been identified to play a role in these process. Of these soluble factors, the only well-characterized one is interleukin (IL)-15 and its role as a positive regulator of NK cell production and proliferation. Little is known, however, about factors that negatively regulate NK cell homeostasis. Transforming growth factor-beta (TGF-β) is known to be a potent immunosuppressing cytokine that affects many cell types. Our lab has found that functional inactivation of TGF-β receptor (TGF-βR) signaling in a CD11c^{dnR} transgenic mouse model results in an increase in NK cell numbers, suggesting that TGF-β is a negative regulator of NK cell production and proliferation. Using bromodeoxyuridine labeling method, I observed significant increase in proliferation rates of NK cells in CD11c^{dnR} mice compared to littermate controls. Subsequently, I conducted a set of in vitro experiments using Annexin V and PI labeling and different IL-15 treatment conditions to determine the difference in IL-15 requirement for survival and proliferation between wild type and TGF-β-resistant NK cells. My findings demonstrate that in the absence of TGF-βR signaling, NK cells proliferate more vigorously and the threshold for IL-15 required for survival is reduced. These findings denote a previously undescribed function of TGF-β in regulating NK cell homeostasis by inhibiting NK cell proliferation and increasing the threshold of IL-15 required for NK cell survival and proliferation.

INTRODUCTION

The immune system is composed of two arms - the innate and adaptive immunity - which function to protect the host from potentially harmful agents such as bacteria, viruses, parasites, and abnormal host cells (Murphy et al., 2008). Traditionally, innate immunity refers to a variety of short-lived myeloid and lymphoid cells that include granulocytes, mast cells, NK cells, dendritic cells, and macrophages. These cells carry out a rapid, non-specific response to pathogen invasion (Murphy et al., 2008). These cells perform their immune function through a limited repertoire of germline-encoded receptors (Vivier et al., 2011). In contrast, adaptive immunity refers to slow, specific responses carried out by two types of lymphocytes, T and B cells, which express a large repertoire of antigen receptors produced by site-specific somatic recombination on the T cell receptor and antibody/B cell receptor (Murphy et al., 2008; Vivier et al., 2011). Recent findings characterizing NK cell receptors, however, demonstrate that NK cells express antigen-specific receptors and memory-like qualities resulting in increased specificity and more robust secondary responses to infections (Sun et al., 2009). Taken together, these findings suggest that NK cells represent an evolutionary bridge between the innate and adaptive immunity. The role of NK cells as a first line of defense against tumors and a diverse range of pathogens further conclusively demonstrates the significance of NK cells and the importance of further studies on NK cells.

NK cells - what is in the name?

NK cells are large granular lymphocytes that are classically associated with the innate immune system because of their role in providing a rapid, non-specific response to pathogen invasion and cancer. NK cells are identified by the expression of molecular markers such as CD122, NK1.1, CD49b (DX5), NKp46, CD11c, and a vast repertoire of inhibitory and activating receptors (Spits and Lanier, 2007). Since these receptors are also expressed by other cell types, however, unambiguous NK cell identification can only be accomplished by the expression of a combination of these receptors rather than by the sole expression of one of these receptors. To this end, NK cells can be identified as $CD3^-NK1.1^+CD122^+$ cells in C57BL/6 mice or $CD3^-DX5^+CD122^+$ cells in other strains

that do not express NK1.1 such as Balb/c strain. The inclusion of CD3$^-$ cells is necessary to exclude natural killer T cells, the only other well-characterized cell type that is NK1.1$^+$CD122$^+$ (Di Santo, 2006). However, recently NKp46 has been identified as a specific marker for all NK cells (Walzer et al., 2007).

True to their name, NK cells are involved in defense against viral and bacterial infections and are specialized in killing infected cells and tumor cells by cytolytic and proinflammatory mechanisms (Lodoen and Lanier, 2006). Although initially believed to perform nonspecific killing, NK cells have been found to employ a combination of inhibitory and activating receptors to perform killer effector functions (Lodoen and Lanier, 2006; Raulet, 2004; Yokoyama, 2008).

Originally, the missing-self hypothesis was proposed as a mechanism by which NK cells identified target cells by the absence of major histocompatibility complex (MHC) class I receptors (Karre et al., 1986). MHC class I receptors are expressed by most cell types and typically present a self-antigen that marks the cell as self (Murphy et al., 2008). The principle behind the missing-self hypothesis is that inhibitory receptors expressed on the surface of NK cells specifically recognize self MHC class I molecules. If NK cells encounter a cell and do not recognize MHC class I molecules, then they will carry out killer effector functions on this cell (Ljunggren, 1990; Raulet, 2004}. These inhibitory receptors include Ly49 receptors in mice, killer immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LIRs) in humans, and CD94-NKG2 receptors in both species. Lack of, or otherwise abnormal, MHC Class I expression is often associated with infection or transformation (Raulet, 2004). The mechanism behind the alteration of MHC class I expression due to infection, transformation, or other forms of stress is not perfectly understood, but it is thought that viral infection often results in the downregulation of MHC Class I molecules as a means of immune evasion from CD8$^+$ cytotoxic T cells and that the missing-self recognition by NK cells was developed as a counter-response by the immune system (Raulet and Vance, 2006).

The missing-self hypothesis has been refined after studies have demonstrated that, similar to other lymphocytes, NK cells must be triggered by a combination of activating and costimulatory receptors to become activated and lyse target cells or

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produce effector cytokines (Lanier, 2006; Raulet and Vance, 2006). To this end, stimulatory receptors expressed on the surface of natural killer cells such as NKG2D, NKp46, NKp44, and NKp30 have been identified to recognize ligands which are specifically upregulated by tumor cells, virally infected cells, and stressed cells (Diefenbach and Raulet, 2003; Raulet, 2004).

NK cells have also been found to employ other activating receptors that directly recognize viral proteins (Diefenbach and Raulet, 2003; Lodoen and Lanier, 2006). This mode of identification is exemplified by the Ly49H receptor which recognizes the m157 mouse cytomegalovirus (MCMV) viral protein (Raulet, 2004). NK cells from C57BL/6 mice normally express Ly49H receptors and are resistant to MCMV infection. In contrast, BALB/c mice are deficient in Ly49H receptor expression and are susceptible to MCMV infection (Cheng et al., 2008; Fodil-Cornu et al., 2008). These findings demonstrate the significance of the role of NK cells in providing protection against various pathogens by performing specific killing mediated by direct recognition of pathogen-associated molecular patterns (PAMPs).

NK cells can also be activated by the upregulation of NKG2D ligands or cytokines that are the products of activated macrophages, dendritic cells, and other myeloid cells (Lodoen and Lanier, 2006; Raulet, 2004). Cells can respond to pathogen infection by the recognition of PAMPs through pattern recognition receptors such as toll-like receptors (TLRs). PAMPs include bacterial lipopolysaccharides (LPS), flagellin, lipoteichoic acid, peptidoglycan, and nucleic acid variants normally associated with viruses. PAMPs recognition activates cells and induces effector functions that ranges from proinflammatory, cell-mediated responses to humoral, antibody-mediated responses (Murphy et al., 2008). One of the proinflammatory, cell-mediated responses that may result from PAMPs recognition involves activating NK cells by producing cytokines such as tumor necrosis factors (TNF), type I interferon (IFN), interleukin (IL) - 12, and IL-18 (Lodoen and Lanier, 2006; Raulet, 2004). T cells and other cell types are also known to produce IL-2, IL-15, and other members of the common-gamma chain cytokine family which affect NK cell function, development, and proliferation (Meazza et al., 2011).

NK cell activation results in two main effector mechanisms that are used to control infection or kill infected or abnormal cells -the secretion of proinflammatory cytokines and direct lysis of cells (Lodoen and Lanier, 2006). Proinflammatory cytokines produced include IFN-γ and TNF-α (Lodoen and Lanier, 2006). The immune system uses inflammation as a means of simultaneous destruction and healing of tissue resulting in clearance of infections. The release of proinflammatory cytokines recruits and activates cells such as granulocytes which release destructive inflammatory soluble factors and promotes a cell-mediated immune response involving T helper type 1 cells, CD8$^+$ T cells, and macrophage activity (Murphy et al., 2008). The lytic function of NK cells is mediated by the release of perforin and granzymes which attack and compromise the cell membrane of target cells (Lodoen and Lanier, 2006; Murphy et al., 2008). NK cells come preloaded with abundant granzyme A and mRNA encoding perforin and granzyme B which are translated upon NK cell activation. This pre-existing pool of granzyme A and mRNA for granzyme B and perforin allows for the rapid response of NK cells to infected or abnormal cells (Fehniger et al., 2007).

Recent studies have also demonstrated the capacity of NK cells to further shape and regulate the immune response generated to infection by secretion of immune-suppressive cytokines such as IL-10, growth factors such as granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and IL-3, and chemokines such as CCL2, CCL3, CCL4, CCL5, CXCL1, and CXCL8 (Vivier et al., 2011). Subsets of NK cells with increased specialization towards cytotoxic or proinflammatory effector functions have also been identified based on the expression of various surface markers such as CD11b, CD27, and CD127 (Di Santo, 2008).

How do NK cells develop?
NK cells are bone-marrow derived and share a common lymphoid progenitor with T and B cells (Hesslein and Lanier, 2011). NK cell development primarily takes place in the bone, but subsets of NK cells have also been reported that develop in the thymus as well as fetal/newborn liver (Di Santo, 2006, 2008; Hesslein and Lanier, 2011; Sun and Lanier, 2011). Thymic NK cells are identified by expression of CD127 and have

increased cytokine-producing capacity and reduced cytotoxicity (Vosshenrich et al., 2006). Fetal/newborn liver NK cells are identified by the expression of TRAIL while lacking DX5 and Ly49 receptors and have been associated with providing immune surveillance towards stressed or transformed cells during the fetal/newborn stage of life (Takeda et al., 2005).

Models for the stages of NK cell development have been described based on the expression of various cell surface markers as illustrated in **Figure 1**. Expression of CD122 by a non-stromal bone marrow cell has been correlated with commitment to the NK lineage and is referred to as an NK cell precursor (NKP), although it does not yet express NK cell lineage-specific markers (Di Santo, 2006; Sun and Lanier, 2011). CD122 refers to the IL2Rβ chain which is necessary for IL-15 recognition. IL-15 is required for NK cell generation and survival (Colucci et al., 2003; Hesslein and Lanier, 2011). Mice lacking IL-15 or its unique receptor component are highly deficient in NK cells (Jamieson et al., 2004). Mice models deficient in the expression of the common gamma chain, which is another part of the IL-15 receptor complex, such as gamma chain knockout mice have no NK cells (DiSanto et al., 1995).
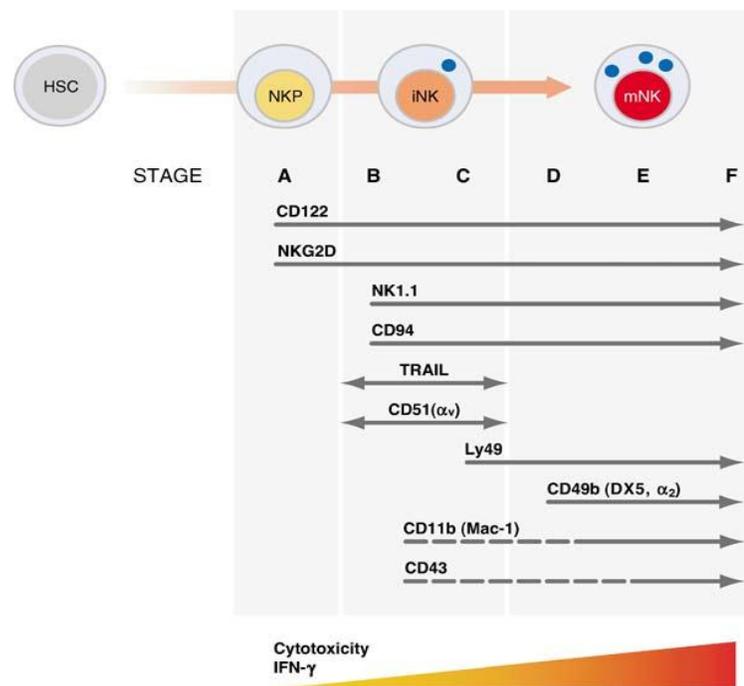
Figure 1: Phenotypic markers of developing NK cells. NK precursors (NKP, *stage A*) are characterized by CD122 and NKG2D expression, but they lack other NK cell markers. Immature NK cells (iNK) upregulate NK1.1 and CD94 and transiently express TRAIL and CD51 (*stages B and C*). Ly49 receptor repertoires and DX5 are relatively late markers of NK cell differentiation expressed by mature NK cells (mNK,*stages D and E*). CD11b and CD43 expression increase as NK cells differentiate, although effector functions do not depend on upregulation of these markers (Di Santo, 2006).

Similarly, mice with deficiencies in IL-15 production such as IL-15 knockout mice have dramatically reduced NK cell numbers (Kennedy et al., 2000). This demonstrates the importance of CD122 in NK cell development and survival and is a likely reason for its early expression. NKG2D is also expressed in the NKP stage (**Figure 1**). The transcriptional factors Ets-1, Id2, Ikaros, and PU.1 have been identified in playing an important role in the generation of NKP through a mechanism that remains to be elucidated (Barton et al., 1998; Scott et al., 1994; Yokota et al., 1999). This NKP stage denoted by CD122 and NKG2D has been associated with being the earliest-committed NK cell progenitor. Recent studies, however, have identified a population of previously believed to be common lymphoid progenitors that are $CD27^+CD244^+C\text{-}KIT^+IL\text{-}7R\alpha^+$ but have down regulated Flk2 and are $CD122^-$. Researchers claim that this is a population of committed NK cell progenitors. If true, these cells may be the earliest committed NK cell progenitor cells yet to be identified (Fathman et al., 2011).

The next stage of NK cell development is the immature NK (iNK) cell stage and involves expression of NK1.1 and CD94. The transcriptional factors Gata-3, IRF-2, and T-bet are important in the development and maturation of iNK (Samson et al., 2003; Taki et al., 2005; Vosshenrich et al., 2005b).

Further maturation from iNK to mature NK (mNK) cells is characterized by the expression of DX5 and a vast repertoire of Ly49 receptors (Di Santo, 2006). Mature NK cells progress through stages D, E, then F as they differentiate and upregulate CD11b, CD43, and various other activating and inhibitory receptors before reaching terminal maturation. The functional differentiation leading to this terminal maturation is influenced by the transcriptional factors CEBP-γ, MEF, and MITF (Vosshenrich et al., 2005b). Upon reaching terminal maturation at the F stage, NK cells leave the bone marrow and home to various peripheral tissues. Mature NK cells are found in the bone marrow, spleen, blood, liver, lung, and lymph nodes where their numbers are tightly controlled by homeostasis (Di Santo, 2006; Sun and Lanier, 2011).

NK cell precursors and immature NK cells have also been found in other tissues such as spleens and lymph nodes that suggest that NK cells may also develop in peripheral tissues, and recent studies have identified NK cell subsets in various

peripheral tissues (Di Santo, 2008). One such case is with NK22 cells in the gut which are distinct from conventional NK cells in function and expression of many NK cell surface markers except for NKp46 (Colonna, 2009; Spits and Di Santo, 2011). The designation of these cells as NK cells begs the question of what is necessary for cells to be classified as NK cells.

How are NK cells maintained?

Not much is known about the actual mechanism of maintaining NK cell numbers in the periphery compared to other cell types such as T cells. The main factor known for playing a major role in peripheral NK cell homeostasis is IL-15 (Jamieson et al., 2004; Ranson et al., 2003). IL-15 is a member of the common-gamma chain cytokine family. As depicted in **Figure 2**, members of this cytokine family signal through a receptor complex consisting of an alpha chain complexed with the common-gamma chain. This receptor complex then mediates signal transduction through JAK1 and JAK3 and different STAT molecules (Meazza et al., 2011). IL-15 and IL-2 share the IL-2 receptor (IL-2R) β chain. Both IL-2 and IL-15 have been demonstrated to promote NK cell proliferation and function which is attributed to this shared signaling pathway (Meazza et al., 2011). However, studies of mice defective in IL-2, IL-2Rα, and IL-2Rβ have shown that IL-2 is not necessary in NK cell development, but IL-2Rβ is required mainly because of its role in IL-15 signaling (Cooper et al., 2002; DiSanto et al., 1995; Ranson et al., 2003; Vosshenrich et al., 2005a).

The role of IL-15 as a proliferative and survival factor in NK cell homeostasis involves its role in mediating the maintenance of the anti-apoptotic factor bcl-2, protecting mature NK cells from apoptosis (Carson et al., 1997). This is accomplished by the inhibition of Bim expression by a variety of mechanisms involving Erk-1/2 phosphorylation or the phosphatidylinositol-3-OH kinase-dependent inactivation of the transcription factor Foxo3a (Huntington et al., 2007). IL-15 also promotes NK cell survival by upregulation of Mcl-1 (Huntington et al., 2007). Adoptive transfer experiments and bromodeoxyuridine (BrdUrd) labeling have been used to demonstrate that splenic NK cells incorporate BrdUrd much more slowly than previously anticipated

with a half-life of about 17 days compared to the previous notions of the half-life only being about 1 day (Jamieson et al., 2004). These studies have also demonstrated that although IL-15 is necessary for survival of NK cells and induces proliferation of NK cells, it is not necessary for homeostatic proliferation of NK cells (Jamieson et al., 2004). Further adoptive transfer experiments have demonstrated that NK cells typically have a half-life of about one week but rapidly disappear within 48 hours in the absence of IL-15 (Sun and Lanier, 2011).

Interleukin-inducible genes
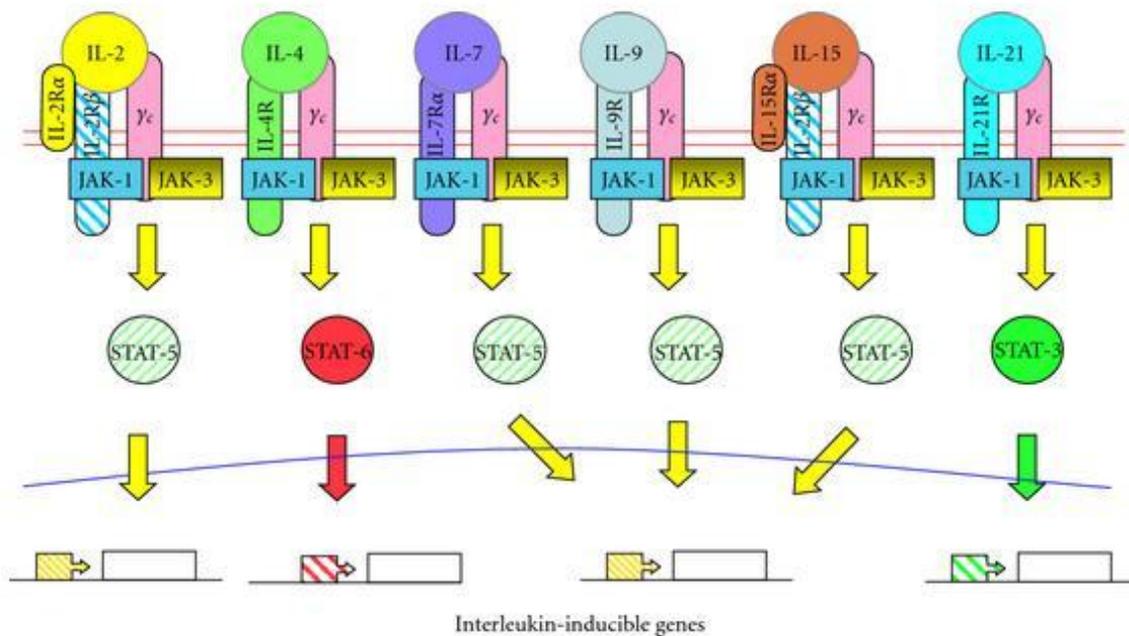
Figure 2: Family of the common cytokine-receptor $γ_c$. Each cytokine binds to a specific α chain, which forms a receptor complex with the $γ_c$. In case of IL-2 and IL-15, trimeric high affinity complexes, which include common IL-2Rβ and $γ_c$ chains, can be formed. Each receptor complex mediates signal transduction through JAK1 and/or JAK3 and different STAT molecules. Tyr-phosphorylated STAT dimers regulate transcription of specific cytokine-sensitive genes. (Meazza et al., 2011).

Other members of the common-gamma chain cytokine family have also been identified in playing roles in NK cell homeostasis, differentiation, and function. To this end, IL-4 has been shown to inhibit NK cell function by negatively influencing NK/dendritic cell cross-talk and T helper type 1 functions while favoring T helper type 2 responses (Marcenaro et al., 2005). IL-7 has also been shown to play a role in NK cell

differentiation by supporting the differentiation of hematopoietic stem cells into lymphoid progenitor cells and by promoting the development of specific NK cell subsets, although these functions remain to be further elucidated (Cella et al., 2010; Satoh-Takayama et al., 2010). Finally, studies have shown that although IL-21 has been shown to promote NK cell proliferation and survival, it may antagonize IL-15-induced proliferation and survival of NK cells by promoting the terminal differentiation of NK cells, but this remains poorly understood (Brady et al., 2010; Kasaian et al., 2002).

NK cells and TGF-β what is the relationship?

TGF-β is a member of the transforming growth factor-beta superfamily which consists of members such as TGF-β 1, TGF-β 2, TGF-β 3, bone morphogenetic protein, inhibins, and activins (Li and Flavell, 2008). As depicted by **Figure 3**, members of the TGF-β superfamily signal through the TGF-βRI and TGF-βRII receptors which dimerize and function as a serine-threonine kinase upon ligand binding, resulting in the phosphorylation of Smad2, followed by a signal cascade (Li and Flavell, 2008).
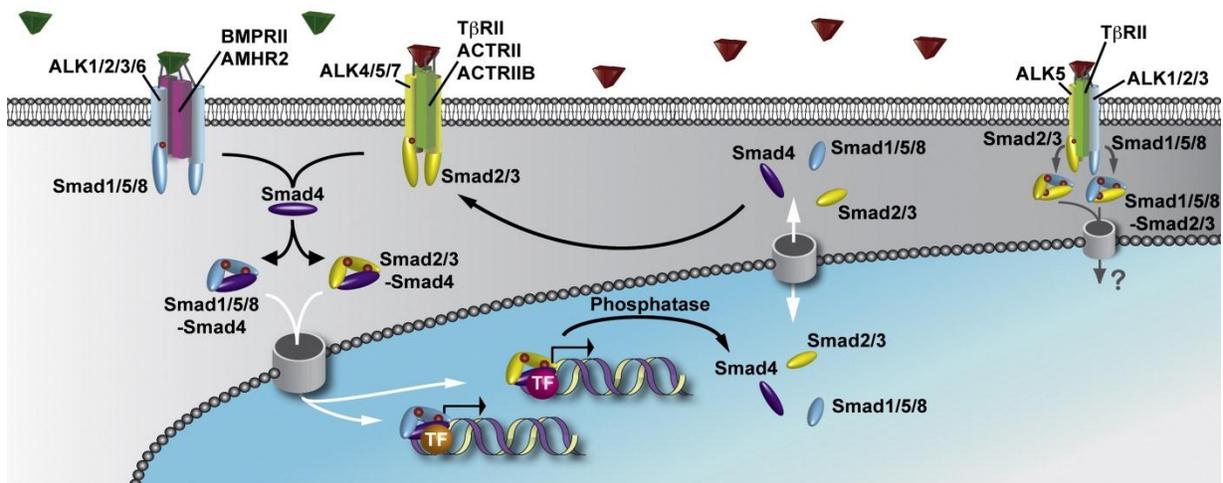
Figure 3: <u>Molecular Mechanism of TGF-β Superfamily Signaling</u>. Heterotetrameric receptor complexes are activated by ligand dimers (red and green triangles) and phosphorylate downstream effectors, the R-Smads (Smad1/5/8/2/3). Phosphorylated R-Smads then form complexes with each other (right-hand side of figure) and/or the co-Smad, Smad4 (left-hand side of figure), and accumulate in the nucleus to regulate target gene transcription. The function of the mixed R-Smad complexes in the nucleus is not yet clear. Transcription factors (TF) cooperate with Smad complexes on DNA. Nonphosphorylated monomeric Smads shuttle in and out of the nucleus. Multimeric complexes dissociate after R-Smad dephosphorylation in the nucleus and shuttle back to the cytoplasm. This is a receptor activity monitoring system (Wu and Hill, 2009).

TGF-β is a versatile immunoregulatory cytokine secreted by many cell types with potent immunosuppressing functions as well as proinflammatory functions (Murphy et al., 2008). Its effects on regulating T cells in the adaptive immunity have been well studied, and TGF-β is known to suppress differentiation and proliferation of T helper type 1 and 2 cells while promoting differentiation and proliferation of T regulatory and T helper type 17 cells (Li and Flavell, 2008). However, little is known about the specific role of TGF-β on the innate immunity and innate immune cells such as NK cells.

Transforming growth factor-beta has been identified as a negative regulator of NK cell homeostasis is (Laouar et al., 2005). Our lab has demonstrated that CD11c[dnR] mice with functional inactivation of TGF-βR signaling due to an overexpression of a dominant-negative truncated form of the TGF-β receptor type II (TGF-βRII) controlled by the CD11c promoter have an increased peripheral NK cell pool (**Figure 4**, left panel). NK cells typically constitute about 3% of the lymphocyte population in the spleen of wild type mice. However, NK cells constitute about 10% in CD11c[dnR] mice (**Figure 4**, right panel).
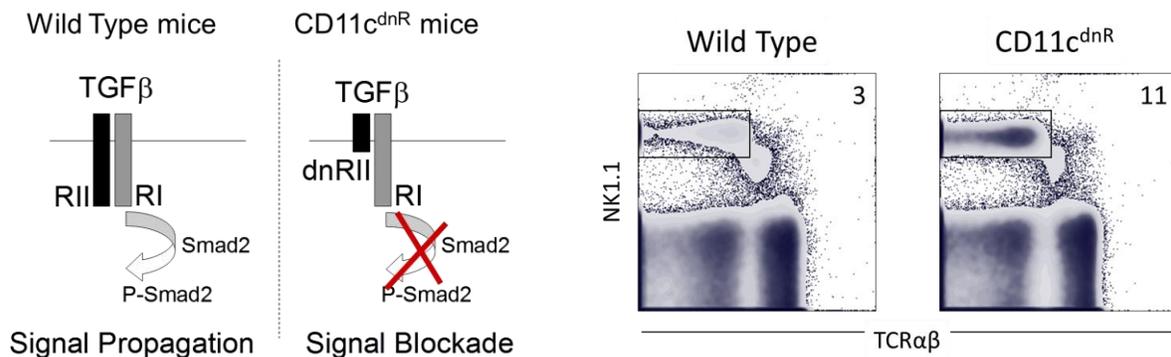
Figure 4: Characterization of CD11c[dnR] mice. Left: CD11c[dnR] mice dominantly express a nonfunctional TGF-β receptor type II (TGF-βRII) in which the cytoplasmic domain of the receptor has been truncated. This transgene is located on the CD11c promoter resulting in the functional inactivation of TGF-βR signaling in CD11c^{+} cells in these mice. Right: Functional inactivation of TGF-β signaling results in an increase in NK cell numbers in the periphery. Splenic NK cells increase from 3% of splenic lymphoctyes in wild type C57BL/6 mice to 11% in CD11c[dnR] C57BL/6 mice. Data are representative of increase also observed in other strains. (Laouar et al., 2005)

HYPOTHESIS

We posit that increased numbers of TGF-β-resistant NK cells in CD11c^{dnR} mice is due to increased proliferation and/or survival due to the relief of TGF-β suppression. To this end, I used BrdUrd labeling methods to compare proliferation rates of wild type and TGF-β-resistant NK cells in vivo. TGF-β-resistant NK cells are expected to incorporate more BrdUrd during BrdUrd pulse treatment and lose BrdUrd expression more rapidly during the decay phase of BrdUrd treatment and analysis.

We hypothesized that an increased proliferation in the absence of TGF-β inhibition may be associated with a decrease in the threshold of IL-15 required for survival and proliferation of TGF-β-resistant NK cells. To this end, I used Annexin V and PI labeling in different culture conditions to determine the difference in IL-15 requirements for survival and proliferation of TGF-β-resistant NK cells in vitro. TGF-β-resistant NK cells are expected to survive and proliferate more vigorously than wild type NK cells in all treatment conditions. Moreover, TGF-β-resistant NK cells are expected to survive in IL-15 treatment conditions in which the concentration of IL-15 is too low to promote survival of wild type NK cells.

EXPERIMENTAL STRATEGY

A/ Mouse Model

A1. CD11c^{dnR} mouse model

We used a CD11c^{dnR} mouse model to study the effect of TGF-β on NK cells. CD11c^{dnR} mice have a dominant negative expression of the TGF-β receptor type II (TGF-βRII) in which the cytoplasmic domain of the TGF-βRII receptor has been truncated preventing phosphorylation of Smad2 which is the first step in the TGF-β signaling pathway (Laouar et al., 2005). This transgene is under the control of the CD11c promoter resulting in the expression of this nonfunctional receptor by CD11c^{+} cells such as dendritic cells and natural killer cells (**Figure 5**). Overexpression of this nonfunctional TGF-β receptor results in the functional inactivation of TGF-β receptor signaling in all CD11c^{+} cells as previously described (Laouar et al., 2005).
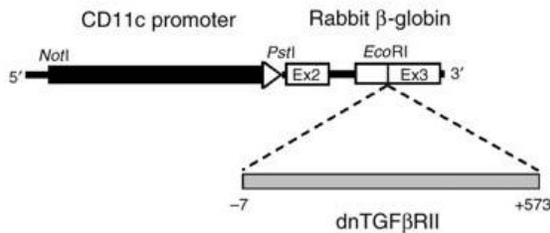
Figure 5: Generation of CD11c^{dnR} mice. CD11c^{dnR} transgene construct. dnTGFβRII, gray box; rabbit β-globin gene exon 3 (Ex3), white box; plasmid CD11c promoter vector, black box.

A2. Generation of bone marrow (BM) chimera mice

Wild type C57BL/6 mice were obtained from the National Cancer Institute and used as recipient hosts for BM chimera mice. Congenic markers were used to differentiate between TGF-β-resistant NK cells from CD11c^{dnR} mice and wild type NK cells from wild type mice. CD11c^{dnR} CD45.1^{+} C57BL/6 mice generated from our lab were used as donors of TGF-β-resistant NK cells and were identified by the expression of CD45.1. Wild type CD45.2^{+} C57BL/6 mice obtained from the National Cancer Institute were used as donors of wild type NK cells and were identified by expression of CD45.2. Recipient hosts were irradiated with 1000 Rads and received an *i.v.* injection of mixed bone marrow cells provided at 1:1 ratio between wild type and CD11c^{dnR} donor cells. Six to

eight weeks post-transfer, composition of splenocytes mice was analyzed to ensure successful reconstitution of NK cells from both donors (**Figure 6**).
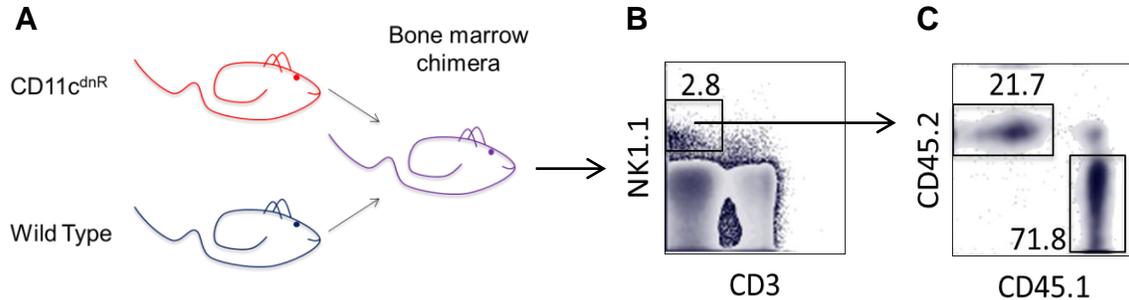

Figure 6: <u>Analysis of mixed BM chimeras</u>. (A) BM cells from wild type and CD11c^{dnR} mice were mixed at a 1:1 ratio and transferred into wild type C57BL/6 recipient mice to generate BM chimera. (B) FACS plot shows the frequency of NK cells generated in mixed BM chimeras 8 weeks post-transfer. (C) FACS plot shows distribution of CD45.1 versus CD45.2 markers among gated NK cells (NK1.1$^+$CD3$^-$).

B/ NK Cell Proliferation

B1. Measuring proliferation using bromodeoxyuridine

Bromodeoxyuridine (BrdUrd) is a thymidine analog that can be incorporated into DNA during the S phase of the cell cycle after administration by intraperitoneal injection or feeding in water. BrdUrd incorporation can then be detected by flow cytometry by using fluorochrome conjugated anti-BrdUrd antibodies (**Figure 7**). Since BrdUrd is incorporated into DNA during the S phase of the cell cycle, detecting BrdUrd incorporation can be used to determine the frequency of cells that have entered the S phase of the cell cycle and are actively proliferating. Monitoring BrdUrd incorporation over time can then be used to compare proliferation rates of cell populations. Similarly, monitoring the loss of BrdUrd expression after cessation of BrdUrd treatment can be used to compare proliferation rates of cell populations (Wilson, 2000). Two methods of BrdUrd treatment and analysis – pulse and decay – were used to compare proliferation rates of wild type and TGF-β-resistant NK cells.
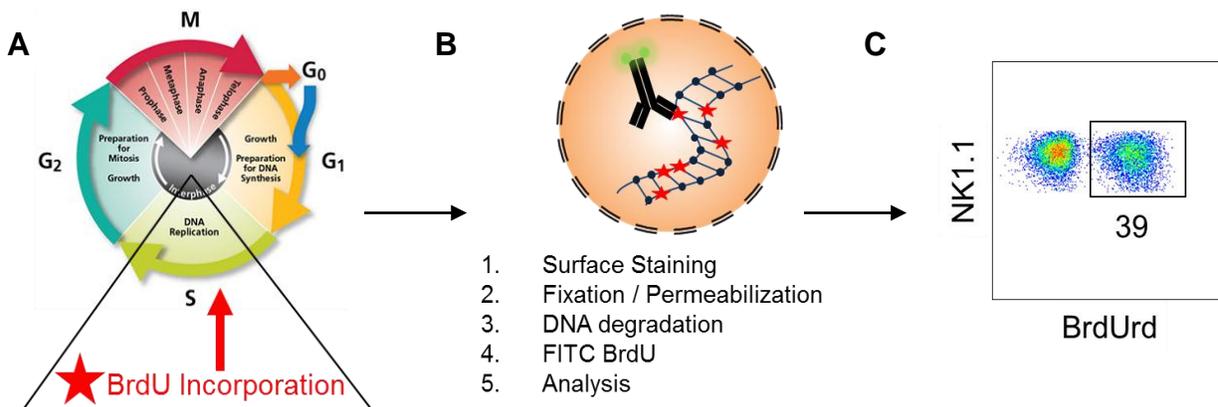
Figure 7: <u>BrdUrd incorporation and revelation.</u> (A) BrdUrd is a thymidine analog that incorporates into the DNA of cells during the S phase of the cell cycle. Picture was modified from http://www.bdbiosciences.com/research/apoptosis/analysis/index.jsp (B) BrdUrd is revealed by flow cytometry after performing surface staining, cell fixation/permeabilization, DNA degradation, and staining of BrdUrd with fluorochrome-conjugated anti-BrdUrd antibody. (C) Cells that are actively cycling are identified by BrdUrd incorporation.

B2. BrdUrd Treatment and Staining Optimization

Prior to performing the actual experiments, BrdUrd treatment and staining was tested by observing BrdUrd incorporation of thymocytes. T cells are known to proliferate vigorously as they go through development in the thymus (Ciofani and Zuniga-Pflucker, 2007). We hypothesized that this robust proliferation would be a good model for testing reagents and procedures for the BrdUrd experiments. Wild type C57BL/6 mice received an *i.p.* injection of 2 mg BrdUrd and administered water with 1 mg/mL BrdUrd overnight (about 16 hours). Mice received another *i.p.* injection of 2 mg/mL BrdUrd 4 hours before sacrificing. Thymuses were harvested and FACS staining was performed using fluorochrome-conjugated anti-CD3, -CD4, -CD8, and -BrdUrd antibodies.
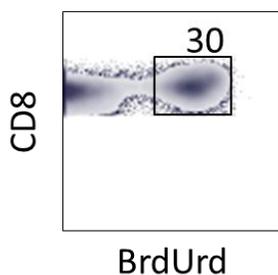
Figure 8: <u>BrdUrd incorporation in thymocytes</u>. Wild type mice were injected *i.p.* with 2 mg BrdUrd for overnight. Subsequently, thymocytes were isolated and detection of BrdUrd was performed according the manufacturer kit. FACS plot shows the frequency of cycling cells among gated $CD4^+CD8^+$ thymocytes. Results are representative of multiple trials with a total of *n*=6 mice.

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The trials on thymocytes demonstrate that BrdUrd treatment and staining procedure planned worked well. In each trial, ~30% of CD3⁻CD4⁺CD8⁺ thymocytes incorporated BrdUrd during the short-term overnight BrdUrd treatment (**Figure 8**).

After having success with thymocytes, BrdUrd treatment was done on wild type C57BL/6 mice and staining was tested for splenic NK cells. Mice received an *i.p.* injection of 2 mg BrdUrd every 12 hours for three days and administered water with 1 mg/mL BrdUrd during the treatment period. Spleens were harvested and FACS staining was performed using fluorochrome-conjugated anti-CD3, -NK1.1, -DX5, and -BrdUrd. Successful staining of BrdUrd incorporation of cycling NK cells prompted continuation with the actual BrdUrd pulse and decay experiments (**Figure 9**).
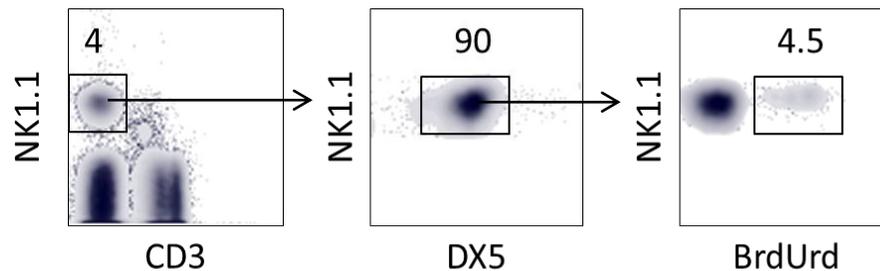
Figure 9: <u>Detection of cycling NK cells using BrdUrd labeling method</u>. Wild type mice were treated with BrdUrd for 3 days as described above. On day 3, mice were sacrificed and NK cells analyzed. FACS plot show the frequency of cycling cells among NK cells as identified by CD3⁻NK1.1⁺DX5⁺ phenotype. This staining and gating procedure was used for BrdUrd pulse and decay experiments.

B3. Methods for BrdUrd Treatment

Two methods of BrdUrd treatment were used to determine rates of proliferation of resting NK cells in the absence of TGF-βR signaling. The pulse method involves continuous BrdUrd treatment and uses the accumulation of BrdUrd⁺ cells as a measure of proliferation. The decay method involves treating with BrdUrd until a significant fraction of cells have incorporated BrdUrd and then monitoring the loss of BrdUrd expression as a measure of proliferation.

<u>BrdUrd Pulse</u>: During the BrdUrd pulse treatment, wild type and CD11c^dnR mice received an *i.p.* injection of 2 mg BrdUrd every 12 hours for 3 days in combination with 1

mg/mL BrdUrd administered in water for a consecutive 10 days (**Figure 10**). Accumulation of BrdUrd staining was analyzed at different time points of this kinetics

BrdUrd Decay: After pulse BrdUrd treatment described above (10 days), wild type and CD11c^{dnR} mice were put back on normal water and BrdUrd staining was measured on different time points after BrdUrd cessation (**Figure 10**).
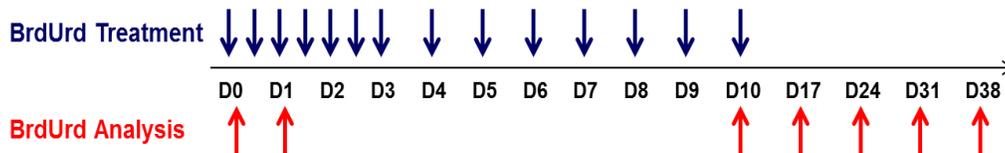

Figure 10: Diagram illustrating BrdUrd Pulse and Cessation. Mice received BrdUrd by *i.p.* injections every 12 hours for the first three days and by water throughout the whole treatment period as indicated by blue arrows. Mice were sacrificed and BrdUrd incorporation was analyzed at different points as indicated by red arrows.

B4. Proof of principle - BrdUrd Pulse and Decay Controls

BrdUrd incorporation by splenic dendritic cells was used as a control during the pulse and decay experiments. We used dendritic cells to set up this method because dendritic cells are endowed with high proliferate and turnover. Their numbers are replenished by a combination of vigorous proliferation by hematopoietic progenitors, monocytes, or local tissue resident cells (Merad and Manz, 2009). Fluorochrome-conjugated anti-CD19, -Class II, -CD11c, and -BrdUrd was used to analyze BrdUrd incorporation in dendritic cells.

As expected, robust proliferation of dendritic cells resulted in massive accumulation of BrdUrd$^+$ dendritic cells that can be observed early in BrdUrd treatment (**Figure 11 A**). As expected, dendritic cells rapidly lost BrdUrd staining within 1 week after cessation of BrdUrd treatment and was almost undetectable 2 weeks later (**Figure 11 B**). These results serve as proof that BrdUrd treatment was successful and that BrdUrd pulse and decay methods described can succesfully translate cell proliferation based on incorporation and loss of BrdUrd.

A

4 hours	24 hours	3 days	7 days	14 days

CD11c

6   29   44   51   65

BrdUrd

B

Day 21   Day 28

CD11c

20   1
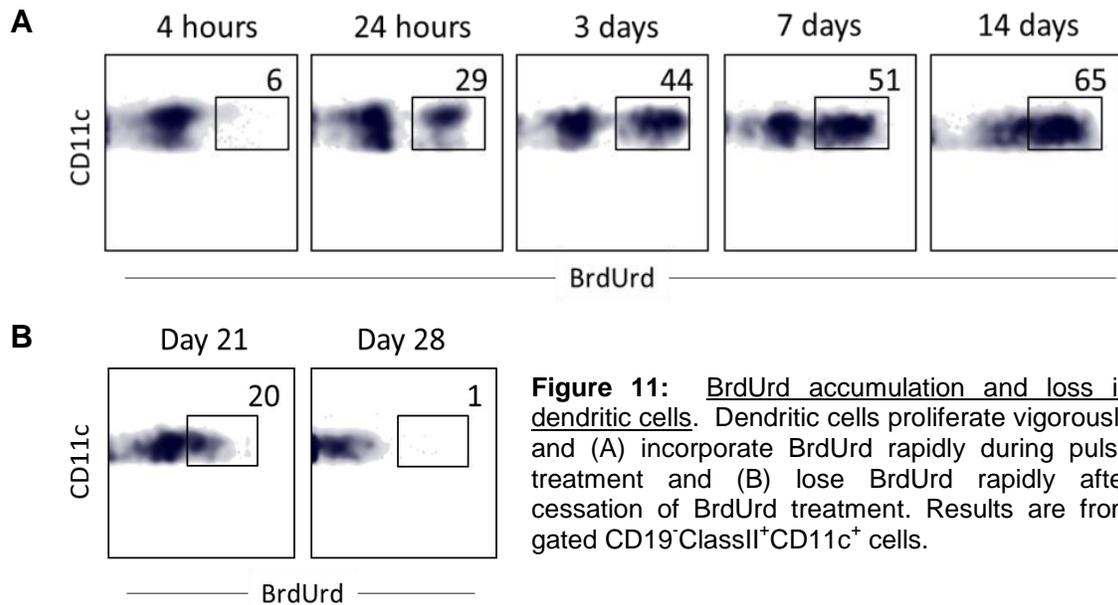
BrdUrd

Figure 11: <u>BrdUrd accumulation and loss in dendritic cells</u>. Dendritic cells proliferate vigorously and (A) incorporate BrdUrd rapidly during pulse treatment and (B) lose BrdUrd rapidly after cessation of BrdUrd treatment. Results are from gated CD19⁻ClassII⁺CD11c⁺ cells.

C/ Cell Death and Survival

C1. Annexin V and Propidium Iodide

Annexin V is a calcium-dependent ligand that binds to phosphatidylserine. Phosphatidylserine is typically expressed in the inside of a cell's cytoplasmic membrane. However, as cells undergo apoptosis, phosphatidyl serine is transferred to the extracellular side of the cytoplasmic membrane (**Figure 12**). Based on this principle, Annexin V staining allows for the identification of apoptotic cells (van Engeland et al., 1996).

Propidium iodide (PI) is a DNA-staining dye. While Annexin V staining allows for the identification of apoptotic cells, it does not allow for differentiation of apopototic and necrotic cells. However, as cells enter the later stages of apoptosis, the semipermeability of their plasma membranes becomes compromised, and PI is able to enter and stain DNA (**Figure 12**). Thus, staining with both PI and Annexin V is necessary to differentiate between apoptotic and necrotic cells (Martin et al., 1995; Span et al., 2002).
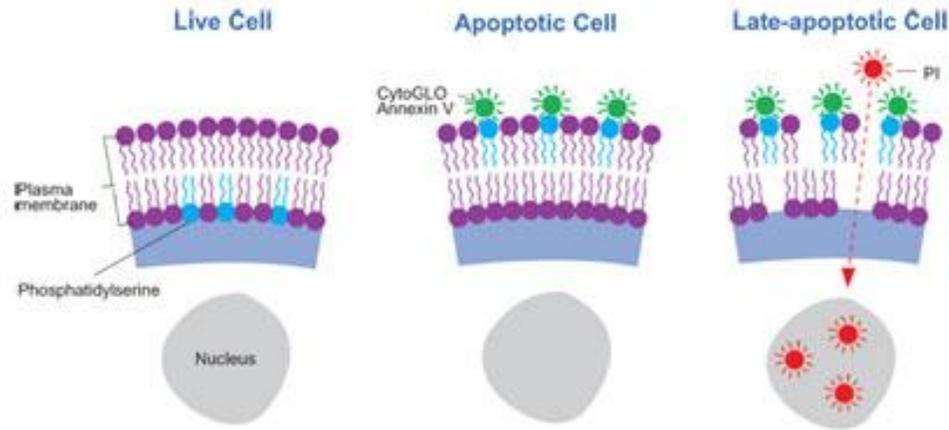
Figure 12: Principle of Annexin V and PI staining. Annexin V binds to phosphatidyl serine which is typically found on the cytoplasmic side of the plasma membrane. Phosphatidyl serine translocates to the external cell surface during the early stages of apoptosis. Staining for only Annexiin V does not allow for the differentiation of apoptotic and late-apoptotic/necrotic cells. Counterstaining with PI allows for the differentiation of apoptotic and late-apoptotic/necrotic cells because PI is not able to enter the cell until the plasma membrane becomes compromised in the later stages of apoptosis. (Image from IMGENEX CytoGLO[TM] Annexin V-FITC Apoptosis Kit description http://www.imgenex.com/apop_kits_list.php?id=76)

C2. Annexin V and PI staining and IL-15 treatment optimization

Prior to performing in vitro culture experiments, Annexin V staining procedures of splenic NK cells were tested and optimized. Splenocytes were harvested from wild type mice and plated at 1 million cells/mL in RPMI media with 5% FCS and 1% PS/Glu. Cultures were incubated overnight in a 37°C incubator.

FACS staining was performed using no buffer, a homemade buffer made in the lab, or buffer from BD Annexin V FITC Apoptosis Kit (catalog number 556547). Annexin V staining worked best with the buffer from the kit and following the procedure provided in the kit (**Figure 13**). For this reason, the BD Annexin V FITC kit staining procedure was used for experiments.
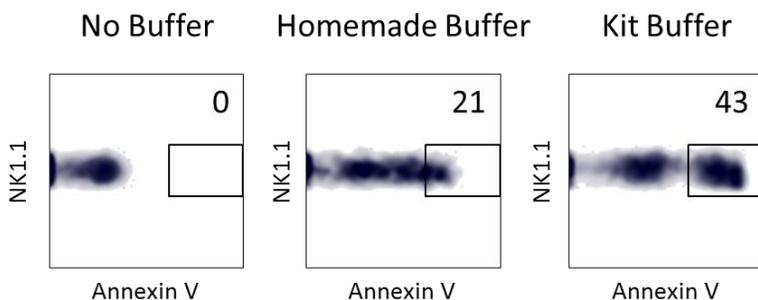
Figure 13: Optimization of Annexin V staining. Annexin V is a calcium-dependent ligand that requires a special buffer containing calcium for successful Annexin V FACS staining. The buffer from the BD Annexin V FITC Apoptosis Kit yielded better results than a homemade buffer.

20

After establishment of a working Annexin V staining procedure, Annexin V and PI counterstaining was tested using the BD Annexin V FITC Apoptosis Kit procedure. Splenocytes were harvested from wild type mice and plated at 1 million cells/mL in RPMI media with 5% FCS and 1% PS/Glu. Cultures were incubated for 3 days in a 37°C incubator. Successful Annexin V and PI staining showed 3 populations of Annexin V⁻PI⁻ live cells, Annexin V⁺PI⁻ apoptotic cells, and Annexin V⁺PI⁺ necrotic cells (**Figure 14**).
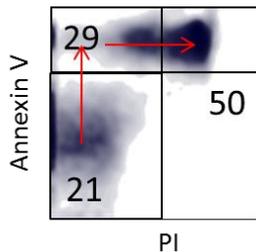

Figure 14: Annexin V and PI optimizaiton. Annexin V counterstaining with PI after 3 days incubation of NK cells. Annexin V⁻ PI⁻ are live cells, Annexin V⁺PI⁻ are apoptotic, and Annexin V⁺PI⁺ are necrotic. FACs plot show gated CD3⁻NK1.1⁺DX5⁺ cells.

Finally, as a final test before performing the actual in vitro culture experiments, IL-15-induced proliferation and survival of NK cells in vitro was tested using the culture conditions that would be used in the actual experiments. Splenocytes were harvested from wild type mice and plated at 1 million cells/mL in RPMI media with 5% FCS and 1% PS/Glu supplemented with 0, 1, 5, 10, 20, or 50 ng/mL IL-15. Cultures were incubated for 7 days at 37°C. As expected, IL-15 is essential in NK cell survival and induces proliferation if the threshold for IL-15 required is reached (**Figure 15**).
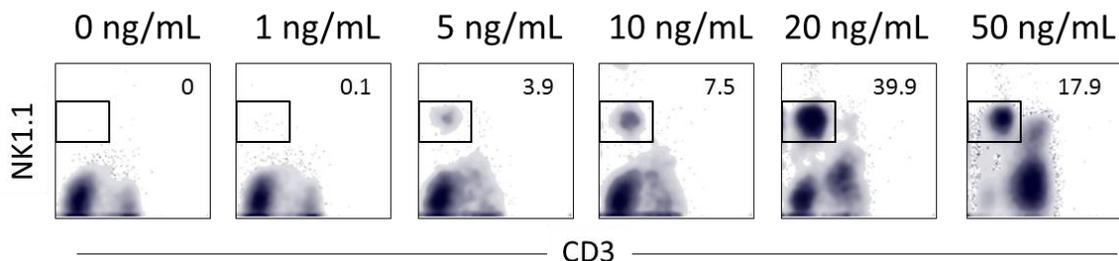
Figure 15: Effect of IL-15 test on Nk cell survival. NK cell culture for 7 days with treatment of 0, 1, 5, 10, 20, or 50 ng/mL IL-15.

C3. NK in vitro Culture Conditions

Splenocytes were harvested from wild type, CD11c^{dnR}, or BM chimera mice and plated at 1 million cells/mL in RPMI media with 5% FCS and 1% PS/Glu. Cultures received 0, 1, 5, 10, 20, or 50 ng/mL of IL-15 every 4 days. Wild type cultures were also treated with 0 or 5 ng/mL of TGFβ. Cultures were incubated in a 37°C incubator until harvested at days 3, 7, 10, and 14 for analysis of NK cell survival.

RESULTS

I. Functional inactivation of TGF-βR signaling in CD11c^{dnR} mice results in robust proliferation of NK cells at steady state
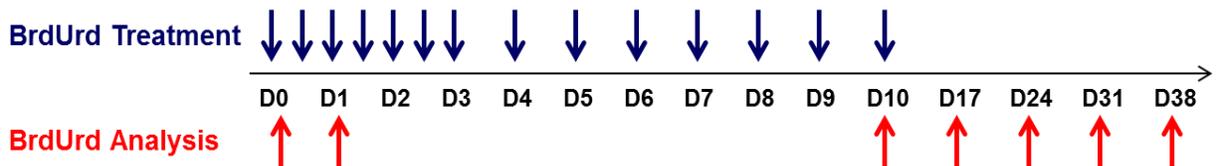
Figure 16: BrdUrd treatment of wild type and CD11c^{dnR} mice. Mice received *i.p.* injections of BrdUrd every 12 hours for the first 3 days of treatment and were administered BrdUrd in water throughout the 10 day BrdUrd treatment duration as indicated by blue arrows. BrdUrd incorporation was measured at 4 hours, 24 hours, 10 days, 17 days, 24 days, 31 days, and 38 days as indicated by red arrows.

A. TGF-β-resistant NK cells incorporate more BrdUrd than wild type NK cells during pulse BrdUrd treatment

In order to study the difference in proliferation rates due to functional inactivation of TGF-βR signaling, bromodeoxuridine (BrdUrd) labeling was used to identify cells that are proliferating and actively cycling. BrdUrd was administered to wild type and CD11c^{dnR} mice by *i.p.* injections and feeding through water for 10 days. BrdUrd incorporation was measured at 4 hours, 24 hours, and 10 days into BrdUrd pulse treatment (**Figure 16**).

BrdUrd$^+$ NK cells were almost undetectable (0.5%) in wild type mice at 4 hours into treatment whereas a small percentage (2.2%) of BrdUrd$^+$ NK cells were detectable in CD11c^{dnR} mice at 4 hours into BrdUrd pulse treatment. Furthermore, the percent of BrdUrd$^+$ NK cells is consistently higher in CD11c^{dnR} mice compared to wild type mice in

each time point during the pulse treatment reaching a final accumulation of 42% BrdUrd$^+$ NK cells in wild type mice compared to 48% BrdUrd$^+$ NK cells in CD11c^{dnR} mice (**Figure 18**). These results suggest that TGF-β-resistant NK cells proliferate more vigorously and thus incorporate more BrdUrd during the pulse treatment. This difference in proliferation rate can be observed even in the early hours of BrdUrd treatment. It is interesting, however, that the difference in BrdUrd$^+$ NK cells in wild type compared to CD11c^{dnR} mice is not as dramatic towards the end of treatment suggesting other factors may be limiting the number of NK cells that can be labeled with BrdUrd.

B. TGF-β-resistant NK cells lose BrdUrd expression more rapidly than wild type NK cells after cessation of BrdUrd treatment

NK cells are known to undergo robust proliferation in the bone marrow during development and maturation prior to homing to peripheral tissues (Di Santo, 2006). Proliferation then slows considerably. To differentiate from BrdUrd incorporation due to robust NK cell proliferation in the bone marrow and homeostatic proliferation in steady state, the BrdUrd continuous pulse treatment was followed with a BrdUrd decay analysis. As cells proliferate after cessation of BrdUrd treatment, they will lose the BrdUrd they have incorporated. BrdUrd treatment ceased after 10 days, and BrdUrd incorporation was measured at days 7, 14, 21, and 28 after cessation of BrdUrd treatment (**Figure 16**).

The percentage of BrdUrd$^+$ NK cells decreases much more dramatically in CD11c^{dnR} mice compared to wild type mice after cessation of BrdUrd treatment. The percentage of BrdUrd$^+$ NK cells dropped to 45% of the starting percentage of BrdUrd$^+$ NK cells after BrdUrd treatment within 1 week in CD11c^{dnR} mice but only dropped to 67% of the starting percentage of BrdUrd$^+$ NK cells in the wild type mice (**Figure 19**). This drop is made even more significant by the higher starting point of BrdUrd$^+$ NK cells in CD11c^{dnR} mice (**Figure 20**). This more rapid loss of BrdUrd$^+$ NK cells in CD11c^{dnR} mice demonstrates an increase in NK cell proliferation upon functional inactivation of TGF-βR signaling.

II. Robust proliferation of TGF-β-resistant NK cells in CD11c^{dnR} mice is cell autonomous

To determine if the increased NK cell proliferation observed in CD11c^{dnR} mice is cell intrinsic, BM chimera mice were generated using bone marrow from wild type and CD11c^{dnR} mice mixed 1:1. BrdUrd pulse and decay experiments were repeated in these BM chimera mice.
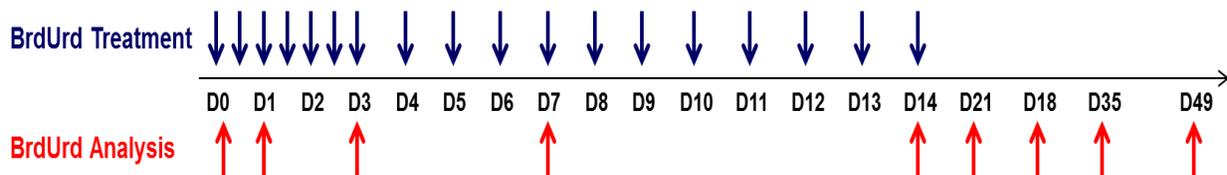

Figure 17: BrdUrd treatment of BM chimera mice. Mice received *i.p.* injections of BrdUrd every 12 hours for the first 3 days of treatment and were administered BrdUrd in water throughout the 14 day BrdUrd treatment duration as indicated by blue arrows. BrdUrd incorporation was measured at 4 hours, 24 hours, 3 days, 7 days, 14 days, 21 days, 28 days, 35 days, and 49 days as indicated by red arrows.

A. Augmented BrdUrd incorporation of TGF-β-resistant NK cells during continuous pulse BrdUrd treatment is cell intrinsic

To study if the increased incorporation of BrdUrd observed during continuous pulse BrdUrd treatment is cell intrinsic, 1:1 wild type to CD11c^{dnR} BM chimera mice were generated in wild type C57BL/6 hosts. BM chimera mice received BrdUrd pulse treatment for 14 days and BrdUrd incorporation was measured at 4 hours, 24 hours, 3 days, 7 days, and 14 days into BrdUrd pulse treatment (**Figure 17**).

A higher percentage of BrdUrd$^+$CD45.1$^+$ NK cells compared to BrdUrd$^+$CD45.2$^+$ NK cells was observed at each time point during BrdUrd pulse treatment in BM chimera mice with a final accumulation of 25.7% BrdUrd$^+$CD45.2$^+$ NK cells compared to 38.7% BrdUrd$^+$CD45.1$^+$ NK cells. More than twice as many BrdUrd$^+$CD45.1$^+$ NK cells were observed in the first three days of treatment compared to BrdUrd$^+$CD45.2$^+$ NK cells (**Figure 21**). Again, this difference in BrdUrd$^+$ cells is not as great towards the end of the treatment period. Increased BrdUrd incorporation of TGF-β-resistant NK cells indicates that NK cell proliferation is increased due to functional inactivation of TGF-βR signaling in a cell intrinsic manner.

B. Augmented loss of BrdUrd expression of TGF-β-resistant NK cells during decay BrdUrd analysis is cell intrinsic

To determine if the rapid loss of BrdUrd observed during BrdUrd decay experiments is cell intrinsic, experiments were repeated in 1:1 wild type to $CD11c^{dnR}$ BM chimera mice generated in wild type C57BL/6 hosts. BrdUrd treatment of BM chimera mice was stopped after 14 days, and BrdUrd incorporation was measured at days 7, 14, 21, 28, and 35 after cessation of BrdUrd treatment (**Figure 17**).

The percentage of $BrdUrd^+CD45.1^+$ NK cells decreased much more dramatically than $BrdUrd^+CD45.2^+$ NK cells in BM chimera mice. The percentage of $BrdUrd^+CD45.1^+$ NK cells dropped by almost 75% by day 7 after cessation of BrdUrd treatment whereas the percentage of $BrdUrd^+CD45.2^+$ NK cells only dropped by 20% (**Figure 22**). Again, this drop is made more significant considering the higher starting value of $BrdUrd^+CD45.1^+$ NK cells compared to $BrdUrd^+CD45.2^+$ NK cells (**Figure 23**). These results indicate that the more rapid loss of $BrdUrd^+$ NK cells in $CD11c^{dnR}$ mice is a cell intrinsic characteristic due to functional inactivation of TGF-βR signaling in NK cells.

III. Functional inactivation of TGF-βR signaling decreases IL-15 requirement for NK cell proliferation and survival in $CD11c^{dnR}$ mice

To examine the difference in IL-15 requirements of wild type and TGF-β-resistant NK cells, splenocytes from wild type and $CD11c^{dnR}$ mice were cultured in different IL-15 treatment conditions. In vitro culture studies demonstrated that TGF-β-resistant NK cells require less IL-15 for survival and proliferation than wild type NK cells (**Figures 24-26**). No NK cells were recovered from wild type splenocyte cultures with 1 ng/mL IL-15. However, treatment with 1 ng/mL IL-15 is enough to rescue and promote the expansion of NK cells in $CD11c^{dnR}$ splenocyte cultures to 8% of splenic lymphocytes recovered. Treatment of wild type splenocyte cultures with 5 ng/mL IL-15 was enough to rescue NK cells and promote a similar level of expansion of NK cells observed in treatment of $CD11c^{dnR}$ splenocyte cultures with 1 ng/mL IL-15. Treatment with 5 ng/mL IL-15

resulted in a more massive expansion of NK cells to 20% of splenic lymphocytes recovered by day 8 and 40% by day 10 in CD11c^{dnR} splenocyte cultures. Little to no difference was observed when splenocytes from either wild type or CD11c^{dnR} mice were cultured at 10 ng/mL of IL-15 with expansion being observed in both cultures. However, expansion in NK cell numbers is observed at an earlier time point in the cultures of CD11c^{dnR} splenocytes. Interestingly, a greater expansion in NK cell numbers was observed in cultures of wild type splenocytes treated with 20 ng/mL of IL-15 with NK cells composing 40% of splenic lymphocytes recovered from wild type splenocyte cultures compared to 17% in CD11c^{dnR} splenocyte cultures (**Figures 24-26**). This suggests that TGF-β-resistant NK cells reach their maximal IL-15 requisite at a lower concentration of IL-15 than wild type NK cells and are detrimentally affected by excess IL-15 after this threshold has been reached. Wild type NK cells similarly fared better than TGF-β-resistant NK cells treated with 50 ng/mL IL-15, although both types were negatively affected by the excess IL-15 and did not do as well as when treated with 20 ng/mL IL-15 (**Figures 24-26**).

Addition of 5 ng/mL TGF-β to all cultures resulted in a drastic decrease in cells recovered from cultures of splenocytes from wild type mice. Essentially no NK cells were recovered from cultures of wild type splenocytes treated with 0, 1, 5, 10, or 20 ng/mL IL-15 as well as 5 ng/mL TGF-β. Treatment with 50 ng/mL IL-15 rescued NK cells but was not able to induce NK cell expansion (**Figures 24-26**).

Annexin V and PI data of CD3$^-$NK1.1$^+$DX5$^+$ cells show that the majority of NK cells (>75%) recovered from cultures that received IL-15 treatment are live. Most cells (~90%) recovered from cultures that received 0 ng/mL IL-15 were apoptotic or necrotic. Addition of 5 ng/mL TGF-β increased the percentage of apoptotic and necrotic cells in all treatment conditions (**Figure 27**). These results serve as an affirmation that the NK cells recovered from the cultures were viable, live cells whose survival is attributed to the IL-15 treatment. These results suggest that TGF-β inhibits IL-15-induced NK cell survival.

IV. Decreased IL-15 requirement for NK cell proliferation and survival in CD11c^{dnR} mice is cell autonomous

To determine if in vitro culture results revealing a decreased threshold for IL-15 required for NK cell survival and proliferation is cell intrinsic, 1:1 wild type to CD11c^{dnR} BM chimera mice were generated. In vitro cultures were replicated suing splenocytes from these BM chimera mice. Cultures of splenocytes from BM chimera mice demonstrate that increased potential for survival is cell intrinsic. CD45.1^{+} cells originate from CD11c^{dnR} donor mice. CD45.2^{+} cells originate from wild type donor mice. NK cell survival was observed in all cultures that were treated with IL-15. Furthermore, expansion of NK cells was observed in cultures treated with 5, 10, 20, or 50 ng/mL IL-15. 70% of NK cells recovered from cultures treated with 1 ng/mL IL-15 at all time points were CD45.1^{+}. Interestingly, except on day 7 when CD45.2^{+} cells compose 67% and 78% of NK cells in cultures treated with 5 or 10 ng/mL IL-15, respectively, little difference was observed between CD45.1^{+} and CD45.2^{+} NK cells in cultures treated with 5 or 10 ng/mL IL-15 with both populations composing about half of the NK cells recovered. A higher proportion of the NK cells recovered from cultures treated with 20, or 50 ng/mL IL-15 were CD45.2^{+} supporting the observation of wild type NK cells doing better in conditions with higher concentrations of IL-15 (**Figures 28-30**).

Similar to results from separate wild type and CD11c^{dnR} splenocyte cultures, Annexin V and PI data of CD3^{-}NK1.1^{+}DX5^{+} cells affirm that the majority of NK cells (>80%) recovered from BM chimera splenocyte cultures are live (**Figure 31**). Taken together, these results suggest that TGF-β inhibits IL-15-induced NK cell survival and proliferation in a cell-intrinsic manner.

DISCUSSION

Two major outcomes from these results are that TGF-β-resistant NK cells have increased proliferation and decreased threshold for IL-15 required for survival.

Increased capacity for NK cell proliferation in the absence of TGF-βR signaling

The BrdUrd pulse and decay results demonstrate that proliferation is dramatically increased in TGF-β-resistant NK cells. The increased proliferation rates of $CD45.1^+$ cells from $CD11c^{dnR}CD45.1^+$ donor cells compared to $CD45.2^+$ cells from wild type $CD45.2^+$ C57BL/6 mice in the BM chimera mice demonstrates that the increased NK cell proliferation observed in $CD11c^{dnR}$ mice is a cell intrinsic characteristic due to functional inactivation of TGF-βR signaling.

NK cells, in part due to their association with the innate immune system, have traditionally been assumed to be short-lived innate immune cells (Jamieson et al., 2004; Sun and Lanier, 2009). However, recent studies have reported that NK cells persist longer than originally conceived (Jamieson et al., 2004). Furthermore, reports of long-lived, homeostatic and memory NK cells that are able to mount secondary responses and contribute to immune memory challenge previously conceived notions of NK cell longevity (Sun et al., 2010; Sun and Lanier, 2009). The persistence of $BrdUrd^+$ NK cells long after BrdUrd treatment cessation supports these findings on the longevity of NK cells.

Studies have demonstrated that it takes about 17 days to label half of the population of splenic NK cells with BrdU (Jamieson et al., 2004). My results of BrdUrd incorporation of wild type NK cells from BrdUrd treatment of wild type and BM chimera mice agree with these findings, with the percentage of $BrdUrd^+$ wild type cells slowly approaching 50% towards the end of BrdUrd treatment. The decreased BrdUrd incorporation in BM chimera mice suggests that extrinsic factors may be inhibiting NK cell survival or proliferation in these mice. Competition for limited resources and space may be one of these factors. Adoptive transfer studies have reported that NK cells undergo massive proliferation upon transfer into a lymphopenic environment and that proliferation drastically decreases upon reconstitution and filling of available space.

This decrease is correlated with an increase in T and B cell proliferation which ultimately constitute a greater proportion of the splenic lymphocyte pool (Jamieson et al., 2004).

The inability to label greater than 50% of NK cells with BrdUrd in CD11c[dnR] mice suggests that there is an increase in NK cell turnover in these mice. Turnover refers to both proliferation as well as death. This increase in turnover rate may be due to the spacial limitations imposed on the cells in the face of increased proliferation or a restriction on available nutrients required to sustain NK cell survival. These findings suggest a role of TGF-β in both suppressing NK cell proliferation while promoting NK cell survival in order to maintain NK cell numbers in steady state conditions.

TGF-β-resistant NK cells proliferate much more rapidly and amount to a much higher percentage of BrdUrd^{+} NK cells during BrdUrd treatment. The percentage of BrdUrd^{+} NK cells drops dramatically within 1 week after cessation of BrdUrd treatment, especially in BM chimera mice. The dramatic decrease in BrdUrd[+] TGF-β-resistant NK cells after cessation of Brdurd treatment may be attributed to a combination of increased NK cell proliferation as well as death in the absence of TGF-βR signaling. A combined theory of increased proliferation as well as death may explain the more dramatic and rapid loss of BrdUrd expression during the BrdUrd decay experiments compared to accumulation during pulse treatment experiments.

The decreased difference in BrdUrd[+] TGF-β-resistant NK cells compared to wild type NK cells during BrdUrd pulse treatment in BM chimera mice suggests that extrinsic factors may also affect NK cell homeostasis in these mice. These factors may include soluble factors produced by CD11c^{+} cells such as dendritic cells transferred from the CD11c[dnR] donor mice. These cells may have increased effector functions due to functional inactivation of TGF-βR signaling that may influence NK cell proliferation. Dendritic cells can activate NK cells by trans-presentation of IL-15 (Huntington et al., 2009). It has also been hypothesized that natural killer cells also possess potential IL-15 trans-presentation and cis-presentation capabilities that allow sustained IL-15 stimulatory effects (Meazza et al., 2011). It is possible that these IL-15 cis- and trans-presentation functions are increased in CD11c[dnR] mice due to functional inactivation of TGF-βR signaling, contributing to the increased NK cell proliferation in CD11c[dnR] mice.

The increased proliferation that is still observed in TGF-β-resistant NK cells in BM chimera mice indicates that, although these extrinsic factors may also contribute to increased NK cell proliferation in CD11c^{dnR} mice, functional inactivation of TGF-βR signaling results in a cell intrinsic increase in NK cell proliferation.

The host being a wild type mouse may also affect the survivability of cells from CD11c^{dnR} donor mice. These factors affecting survivability may also be a reason for the much more dramatic drop in BrdUrd$^+$ TGF-β-resistant NK cell numbers in BM chimera mice compared to CD11c^{dnR} mice at day 7 after cessation of BrdUrd treatment. Further studies using CD11c^{dnR} mice as hosts are necessary to elucidate this phenomenon. Studies involving the transfer of sorted NK cells and using Rag-/-γc-/- mice which are deficient in NK cells and other lymphocytes would help elucidate questions about other extrinsic factors that may influence NK cell proliferation (DiSanto et al., 1995; Garcia et al., 1999).

Functional inactivation of TGF-β signaling decreases the threshold of IL-15 required for NK cell survival and proliferation in vitro

TGF-β-resistant NK cells from CD11c^{dnR} mice demonstrated a higher capacity for survival and proliferation at lower concentrations of IL-15 than wild type NK cells. TGF-β-resistant NK cells were able to survive and proliferate at 1 ng/mL IL-15 whereas little to no wild type NK cells were recovered at this treatment condition. This could be observed in cultures of splenocytes from separate wild type and CD11c^{dnR} mice. This observation is confirmed as a cell intrinsic trait by the cultures of splenocytes from BM chimera mice. Wild type NK cells demonstrated an increased capacity for survival and proliferation as the concentration of IL-15 increased. At 20 ng/mL for TGF-β-resistant NK cells and 50 ng/mL IL-15 for both wild type and TGF-β-resistant NK cells, NK cells appeared to reach a threshold for IL-15 responsiveness. Past this threshold, NK cells either have their survival and proliferation detrimentally affected by the excess IL-15 or they are outcompeted by other cells that also proliferate in response to IL-15 such as CD8$^+$ cytotoxic T cells (Jakobisiak et al., 2011). TGF-β-resistant NK cells reached this threshold of IL-15 at a lower concentration than wild type NK cells. These results

suggest that TGF-β-resistant NK cells would be able to outcompete wild type cells because of their reduced nutrient requirements for survival and proliferation. This may be one reason for the increased splenic NK cell pool in CD11c^{dnR} mice and the larger proportion of CD45.1$^+$ NK cells in the BM chimera mice.

Addition of TGF-β to wild type splenocyte cultures negated the IL-15-induced survival in cultures treated with 0, 1, 5, 10, or 20 ng/mL IL-15. Treatment with 50 ng/mL IL-15 was able to overcome the inhibition imposed by TGF-β but not able to induce expansion of NK cells. These findings further describe a role for TGF-β as an inhibitor of IL-15-induced NK cell survival and proliferation by a mechanism that remains to be identified. These findings also suggest that this mechanism involves antagonistic TGF-βR and IL-15R signaling that necessitates a prerequisite amount of stimulatory IL-15R signaling to overcome the inhibitory TGF-βR signaling in order to promote NK cell survival and inhibition. Lifting of the inhibitory TGF-βR signaling in NK cells of CD11c^{dnR} mice reduces the IL-15 required for survival and proliferation, allowing TGF-β-resistant NK cells to persist in the cultures with 1 ng/mL IL-15 whereas wild type NK cells were unable to survive in the nutrient-scarce environment.

The annexin V and PI results denote that the majority of NK cells recovered from cultures treated with 1, 5, 10, 20, or 50 ng/mL IL-15 were live. In contrast, the majority of NK cells recovered from cultures without IL-15 treatment were apoptotic or necrotic. This observation again demonstrates that IL-15 was able to rescue and promote the survival of NK cells. However, these results reveal flaws in the experimental procedures with regards to the indiscrimination of cell numbers and inability of identifying apoptotic or necrotic NK cells that have down regulated their NK1.1 and DX5 receptors. Higher percentages of apoptotic or necrotic cells were expected in cultures with lower concentrations of IL-15 that correspond with the decreased NK cells recovered from cultures under these treatment conditions. However, there was little to no variability in the annexin V and PI results for cultures that received IL-15 because the annexin V and PI results are gated to the NK cells (CD3$^-$NK1.1$^+$DX5$^+$) recovered. Regardless, the annexin V and PI results for cultures without IL-15 treatment and cultures that received 5 ng/mL TGF-β suggest that the annexin V and PI labeling was

successful. The percentage of apoptotic and necrotic cells also increased upon addition of TGF-β, which was expected considering virtually no NK cells were recovered from these cultures. However, since virtually no NK cells were recovered from these cultures, it is strange that the annexin V and PI data are saying that there are live cells rather than all apoptotic or necrotic cells as was observed in the 0 ng/mL cultures. These results epitomize the problem with indiscrimination towards cell numbers as a misleading factor in the annexin V and PI results obtained. The indiscrimination towards cell numbers involved in this labeling method proves to be a limiting factor that will have to be addressed in future experiments and when performing further analysis of data from these experiments. Still, the annexin V and PI results serve as an affirmation that the NK cells recovered are mostly viable, live cells whose survival is attributed to IL-15 treatment. Experimental procedures must also be refined in future experiments to allow for identification of NK cells that have down regulated their identification markers. Sorting and plating of NK cells instead of whole splenocytes may address some of these issues, but will prevent the characterization of the interplay of other splenocytes in IL-15-induced NK cell survival and proliferation.

Functional inactivation of TGF-β signaling increases NK cell proliferation by reducing the threshold for IL-15 required for survival and proliferation

The findings from the BrdUrd in vivo studies and the in vitro culture studies describe a role for TGF-β in inhibiting IL-15-induced NK cell survival and proliferation. BrdUrd pulse and decay experiments demonstrate that TGF-β inhibits NK cell proliferation in vivo. In vitro cultures with different concentrations of IL-15 demonstrate that TGF-β inhibits IL-15-induced NK cell survival and proliferation by increasing the threshold of IL-15 required for survival and proliferation of NK cells in vitro. Taken together, these findings suggest that the increased proliferation rates of TGF-β-resistant NK cells observed in vivo in the BrdUrd experiments may be attributed to the decreased IL-15 requirements for proliferation due to the functional inactivation of TGF-βR signaling. The increased splenic NK cell pool in CD11c^{dnR} mice may also be attributed to this increased proliferation rate due to decreased IL-15 requirements. This decrease in IL-

15 requirement for survival and proliferation also indicates that the suggested increase in cell death of TGF-β-resistant NK cells from BrdUrd experiments is likely due to spacial limitations rather than nutrient limitations, and that this suggested increase in cell death does not necessarily correlate with a decrease in the long-term survivability and viability of TGF-β-resistant NK cells. These findings denote a previously undescribed function of TGF-β in inhibiting NK cell proliferation by disrupting IL-15 induced survival and proliferation.

TGF-β-resistant NK cells and NK cell therapies

The increased proliferation and decreased IL-15 requirements of TGF-β-resistant NK cells suggests that these NK cells may be more effective as NK cell therapeutic agents than wild type NK cells.

NK cell therapy has been a popular candidate for cancer treatment. Initially, the therapeutic potential for cancer was hindered by the inability of NK cells to effectively clear tumor cells that were identified as self (Geller and Miller, 2011). However, recent studies involving allogeneic transplantation of NK cells have yielded promising results for NK cell immunotherapy against cancer. In these studies, donor NK cells that may express KIR that do not recognize that HLA-class I alleles present on the recipient cells were able to mount an effective anti-leukemia response resulting in efficient lysis of leukemic cells a strong graft versus leukemia effect. The alloreactive NK cells were also able to eliminate residual host dendritic cells, preventing graft-versus-host disease and graft rejection (Moretta et al., 2011).

Even with advancements such as this, NK cell therapies are currently inhibited by the inability of NK cells to persist in large enough numbers and replenish themselves within a new host (Voskens et al., 2010). To this end, studies that involve the development of ex-vivo expansion methods have been performed to try to overcome this barrier (Voskens et al., 2010). The increased capacity of TGF-β-resistant NK cells to proliferate suggests that these cells have a higher potential than wild type NK cells of amounting to a large enough population size to achieve desired therapeutic treatment goals. The decreased IL-15 requirement for survival and proliferation also suggests that

these cells will be able to outcompete endogenous NK cells and persist longer in the host environment. The increased proliferation and decreased IL-15 requirement suggest that TGF-β-resistant NK cells may allow for sufficient in vivo expansion after allogeneic transfer, circumventing current requirements for ex-vivo expansion.

TGF-β is also known to inhibit NK cell effector functions (Terme et al., 2008). For instance, functional inactivation of TGF-βR signaling in NK cells of CD11c[dnR] mice has been correlated with an increased production of IFN-γ by NK cells (Laouar et al., 2005). This suggests that allogeneic transplantation of TGF-β-resistant NK cells would not only pose an increase in therapeutic potential due to a proliferative advantage, but also due to enhanced effector functions. Further studies on differences in effector functions of TGF-β-resistant NK cells would elucidate their true immunotherapeutic potential.

NK cells provide first-line defense against pathogen invasion by viruses, bacteria, and parasites. NK cell protection against infections by viruses such as mouse and human cytomegalovirus, Sendai virus, Influenza A virus, and Ebola virus is associated with production of IFN-γ and other proinflammatory cytokines. NK cell cytotoxicity is also important in protection against mouse cytomegalovirus (Iversen et al., 2005; Loh et al., 2005; Siren et al., 2004; Warfield et al., 2004). The specific role of NK cells in bacterial and parasitic infection is even less understood. However, studies suggest that their cytolytic and proinflammatory functions contribute to defense against infections by *Mycobacterium tuberculosis, Psamodium falciparum, Trypanosoma cruzi, and Shigella flexneri* (Artavanis-Tsakonas et al., 2003; Le-Barillec et al., 2005; Lieke et al., 2004; Vankayalapati et al., 2005). The pluripotent role of NK cells in providing protection against a variety of pathogens suggests that NK cell immunotherapy using TGF-β-resistant NK cells may help alleviate disease burden against a vast range of pathogen infections.

CONCLUSION

Not much is currently known about the soluble factors that regulate NK cell homeostasis. IL-15 is the only well-characterized soluble factor that has been identified to play a role in NK cell homeostasis. Other studies have suggested that other members of the common-gamma chain cytokine family may also regulate NK cell homeostasis, but this remains not well defined (Meazza et al., 2011). Here I present findings that demonstrate the role of TGF-β as a negative regulator of NK cell homeostasis by inhibiting proliferation and increasing the threshold for IL-15 required for NK cell survival and proliferation. Earlier studies have reported a role of TGF-β in dampening NK cell effector functions (Laouar et al., 2005; Terme et al., 2008). Taken together, these findings suggest that TGF-β-resistant NK cells may possess greater immunotherapeutic potential than wild type NK cells because of their increased proliferation, survival, and effector functions.

FUTURE DIRECTIONS

These BrdUrd and in vitro culture studies have established a foundation for further characterization of TGF-β-resistant NK cells. Using Rag-/-γc-/- wild type and CD11c^{dnR} mouse models would help elucidate questions about extrinsic factors that may play a role in NK cell survival and proliferation. Using IL-15-/- mice or other mouse models with deficient IL-15 signaling would clarify the interplay between TGF-β and IL-15 in regulating NK cell survival and proliferation in vivo. Further in vitro studies using IL-15 as well as other cytokines such as IL-12, IL-18, IL-21, IL-2, and IL-4 would also further elucidate the effect of TGF-β on the homeostasis and effector functions of NK cells. Further characterization of the role of TGF-β as an inhibitor of NK cell proliferation, survival, and effector functions would help advance the current knowledge regarding the immunotherapeutic potential of NK cells for cancer and diseases caused by a vast array of pathogens.

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FIGURE LEGENDS

Figure 18: BrdUrd pulse treatment of wild type and CD11c^{dnR} mice. Functional inactivation of TGF-β receptor signaling results in a higher frequency of BrdUrd incorporation in TGF-β-resistant NK cells compared to wild type NK cells at each time point during the pulse BrdUrd treatment. (A) FACS plots gated on CD3$^-$NK1.1$^+$ DX5$^+$ cells. (B) Line graph representation of data from A. Data are representative of n=6 wild type mice and n=6 CD11c^{dnR} mice

Figure 19: BrdUrd decay in wild type and CD11c^{dnR} mice. Functional inactivation of TGF-β receptor signaling results in a more rapid loss of BrdUrd $^+$ NK cells in CD11c^{dnR} mice compared to wild type mice. (A) FACS plots gated on CD3$^-$NK1.1$^+$DX5$^+$ cells. (B) Line graph representing data from A. (C) Line graph of data from A normalized with respects to values at Day 10 when BrdUrd treatment was stopped so that Day 10 equals 100% BrdUrd$^+$ NK cells.

Figure 20: BrdUrd pulse and decay in wild type and CD11cd^{nR} mice. (A) Line graph with combined pulse and decay results from Figure 18 and Figure 19. (B) Line graph of data from Figure 18 and Figure 19 normalized with respects to values at Day 10 when BrdUrd treatment was stopped so that Day 10 equals 100% BrdUrd$^+$ NK cells.

Figure 21: Pulse treatment of BM chimeras. Functional inactivation of TGF-β receptor signaling results in a higher frequency of BrdUrd incorporation in CD45.1$^+$ TGF-β-resistant NK cells compared to CD45.2$^+$ wild type NK cells at each time point during the pulse BrdUrd treatment of BM chimera mice. (A) FACS plots gated on CD3$^-$ NK1.1$^+$DX5$^+$ cells. (B) Line graph representing data A. (C) Line graph of data from A normalized with respects to values at Day 14 when BrdUrd treatment was stopped so that Day 14 equals 100% BrdUrd$^+$ NK cells.

Figure 22: BrdUrd decay in BM chimera mice. Functional inactivation of TGF-β receptor signaling results in a more rapid loss of BrdUrd$^+$ CD45.1$^+$ NK cells than CD45.2$^+$ NK cells in BM chimera mice. (A) FACS plots gated on CD3$^-$NK1.1$^+$DX5$^+$ cells. (B) Line graph representing data from A. (C) Line graph of data from A normalized with respects to values at Day 14 when BrdUrd treatment was stopped so that Day 14 equals 100% BrdUrd$^+$ NK cells.

Figure 23: BrdUrd pulse and decay in BM chimera mice. (A) Line graph with combined pulse and decay results from Figure 20 and Pulse treatment of BM chimeras. Functional inactivation of TGF-β receptor signaling results in a higher frequency of BrdUrd incorporation in CD45.1+ TGF-β-resistant NK cells compared to CD45.2+ wild type NK cells at each time point during the pulse BrdUrd treatment of BM chimera mice. (A) FACS plots gated on CD3-NK1.1+DX5+ cells. (B) Line graph representing data A. (C) Line graph of data from A normalized with respects to values at Day 14 when BrdUrd treatment was stopped so that Day 14 equals 100% BrdUrd+ NK cells.

Figure 22. (B) Line graph of data from Figure 20 and Pulse treatment of BM chimeras. Functional inactivation of TGF-β receptor signaling results in a higher frequency of BrdUrd incorporation in CD45.1+ TGF-β-resistant NK cells compared to CD45.2+ wild type NK cells at each time point during the pulse BrdUrd treatment of BM chimera mice. (A) FACS plots gated on CD3-NK1.1+DX5+ cells. (B) Line graph representing data A. (C) Line graph of data from A normalized with respects to values at Day 14 when BrdUrd treatment was stopped so that Day 14 equals 100% BrdUrd+ NK cells.

Figure 22 normalized with respects to values at Day 10 when BrdUrd treatment was stopped so that Day 10 equals 100% BrdUrd$^+$ NK cells.

Figure 24: FACS plots of separate wild type and CD11c^{dnR} in vitro splenocyte cultures. FACS plots of NK cells recovered from in vitro cultures of (A) wild type and (B) CD11c^{dnR} splenocytes cultured with no TGFβ and 0, 1, 5, 10, 20, or 50 ng/mL IL-15. (C) FACS plot of NK cells recovered from in vitro cultures of wild type splenocytes with 5 ng/mL TGFβ and 0, 1, 5, 10, 20, or 50 ng/mL IL-15.

Figure 25: Line graph representation of wild type and CD11c^{dnR} in vitro splenocyte cultures with data from Figure 24 with days as a function of concentration of IL-15. (A) Combined graph of all days. Graph of (B) day 3, (C) day 8, (D) day 10 (E) day 14.

Figure 26: Line graph representation of wild type and CD11c^{dnR} in vitro splenocyte cultures with data from Figure 24 with IL-15 concentrations as a function of days. (A) Combined graph of all concentrations. Graph of (B) 0 ng/mL, (C) 1 ng/mL, (D) 5 ng/mL, (E) 10 ng/mL, (F) 20 ng/mL, (G) 50 ng/mL IL-15.

Figure 27: Annexin V and PI data of separate wild type and CD11c^{dnR} in vitro splenocyte cultures. Data is gated on CD3$^-$NK1.1$^+$DX5$^+$. Results from in vitro cultures of (A) wild type and (B) CD11c^{dnR} splenocytes cultured with no TGFβ and 0, 1, 5, 10, 20, or 50 ng/mL IL-15. (C) Results from in vitro cultures of wild type splenocytes with 5 ng/mL TGFβ and 0, 1, 5, 10, 20, or 50 ng/mL IL-15.

Figure 28: FACS plots of BM chimera in vitro splenocyte cultures with 0, 1, 5, 10, 20, or 50 ng/mL IL-15. FACS plots of (A) NK cells recovered from in vitro cultures gated to total splenic lymphocytes and (B) the distribution of CD45.1$^+$ and CD45.2$^+$ NK cells gated to CD3$^-$NK1.1$^+$DX5$^+$ cells.

Figure 29: Line graph representation of BM chimera in vitro splenocyte cultures with data from Figure 28 with days as a function of concentration of IL-15. (A) Combined graph of all days. Graph of (B) day 3, (C) day 7, (D) day 10 (E) day 14.

Figure 30: Line graph representation of BM chimera in vitro splenocyte cultures with data from Figure 24 with IL-15 concentrations as a function of days. (A) Combined graph of all concentrations. Graph of (B) 0 ng/mL, (C) 1 ng/mL, (D) 5 ng/mL, (E) 10 ng/mL, (F) 20 ng/mL, (G) 50 ng/mL IL-15.

Figure 31: Annexin V and PI data of BM chimeras in vitro splenocyte cultures. Data is gated on $CD3^-NK1.1^+DX5^+$. Results from in vitro cultures of (A) mixed wild type and $CD11c^{dnR}$ cells from BM chimeras, (B) gated to $CD45.2^+$ wild type cells, and (C) $CD45.1^+$ $CD11c^{dnR}$ cells cultured with no TGFβ and 0, 1, 5, 10, 20, or 50 ng/mL IL-15.

FIGURES

Figure 18 A

Gated on CD3⁻NK1.1⁺DX5⁺ cells

Figure 18 B

Figure 19 A

Day 10   Day 17   Day 24   Day 31   Day 38

Wild Type

NK1.1

42.0   28.0   26.2   20.8   17.4

CD11c^{dnR}

48.2   21.5   13.5   7.83   6.25

BrdUrd
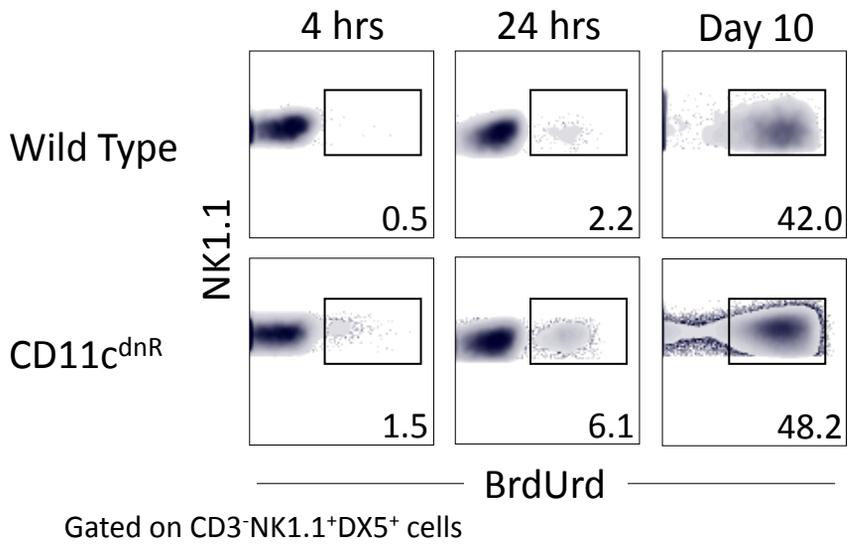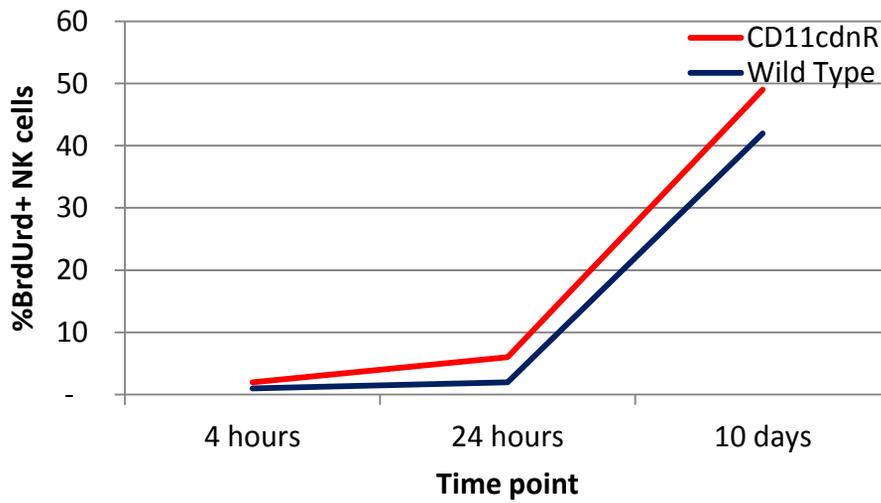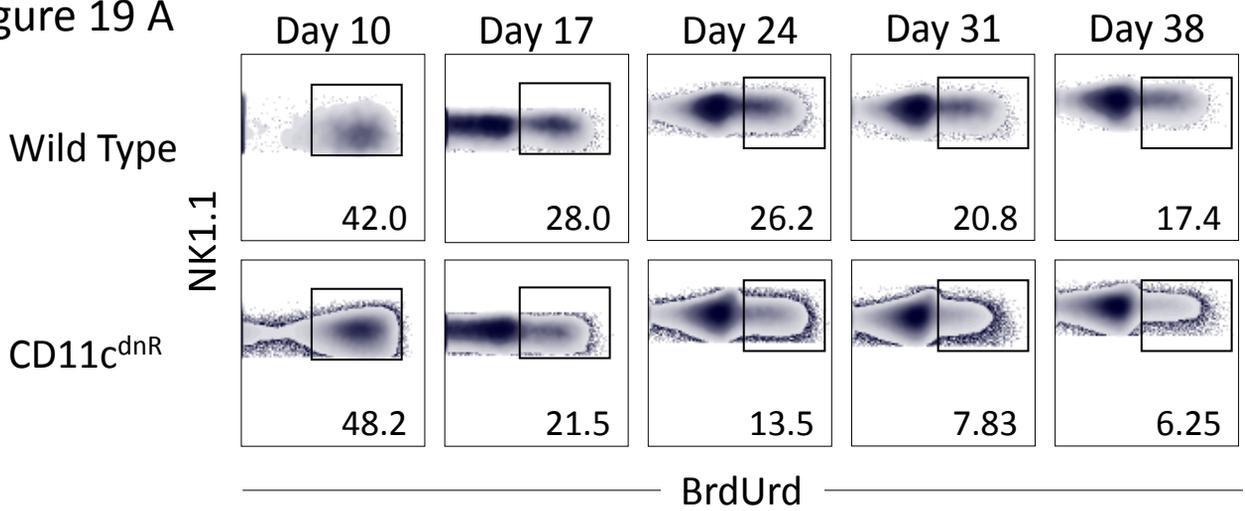
Gated on CD3$^-$NK1.1$^+$DX5$^+$ cells
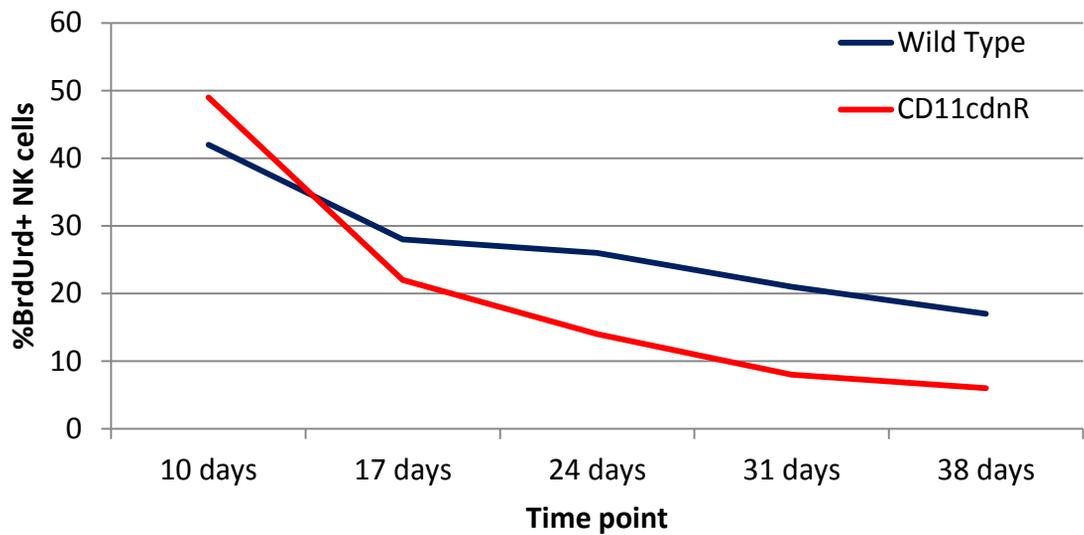
Figure 19 B

Figure 19 C
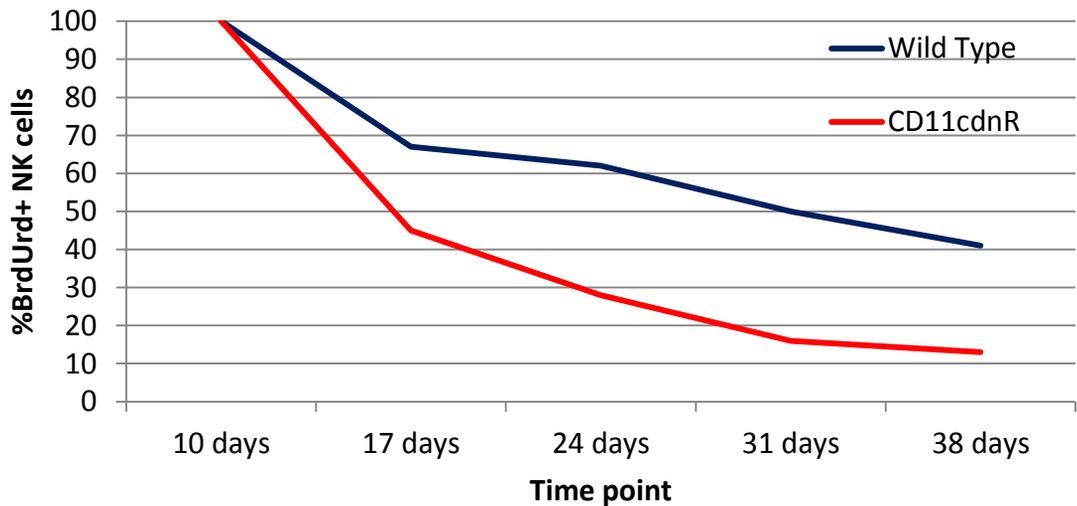
Figure 20 A

Figure 20 B

Figure 21 A

Gated on CD3⁻NK1.1⁺DX5⁺ cells

(table of flow cytometry plots)

	4 hours	24 hours	3 days	7 days	14 days
Mixed donor NK cells (CD45.1⁺ CD45.2⁺)	2.3	7.6	14.6	28.2	35.1
Wild type donor NK cells (CD45.2⁺)	1.6	4.4	8.0	21.8	25.7
CD11c^dnR donor NK cells (CD45.1⁺)	2.6	9.3	17.7	31.9	38.7

BrdUrd

Figure 21 B

Figure 22 A

Mixed donor NK cells (CD45.1$^+$ CD45.2$^+$)

Wild type donor NK cells (CD45.2$^+$)

CD11c^{dnR} donor NK cells (CD45.1$^+$)

Day 14 | Day 21 | Day 28 | Day 35 | Day 49

NK1.1

BrdUrd

Gated on CD3$^-$NK1.1$^+$DX5$^+$ cells

Figure 22 B

Figure 22 C

49
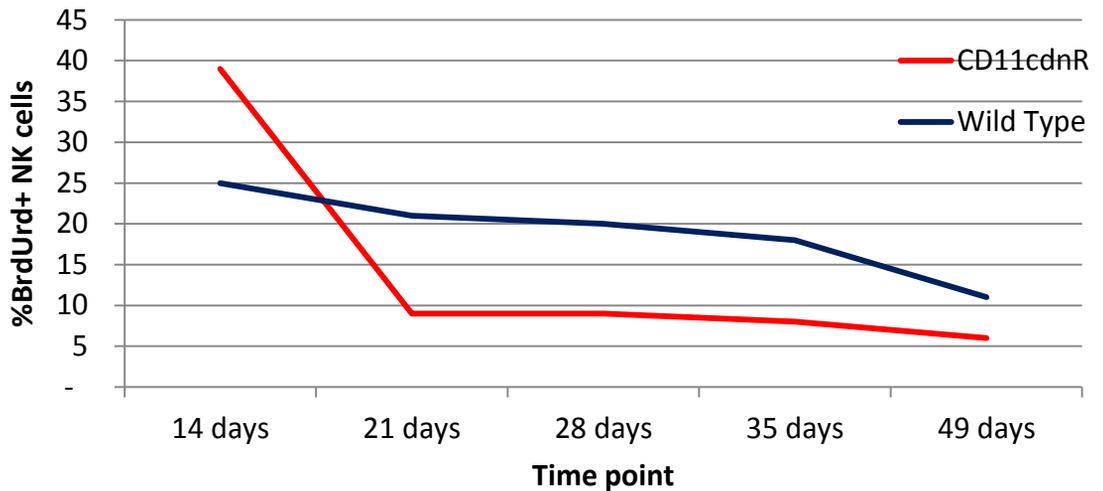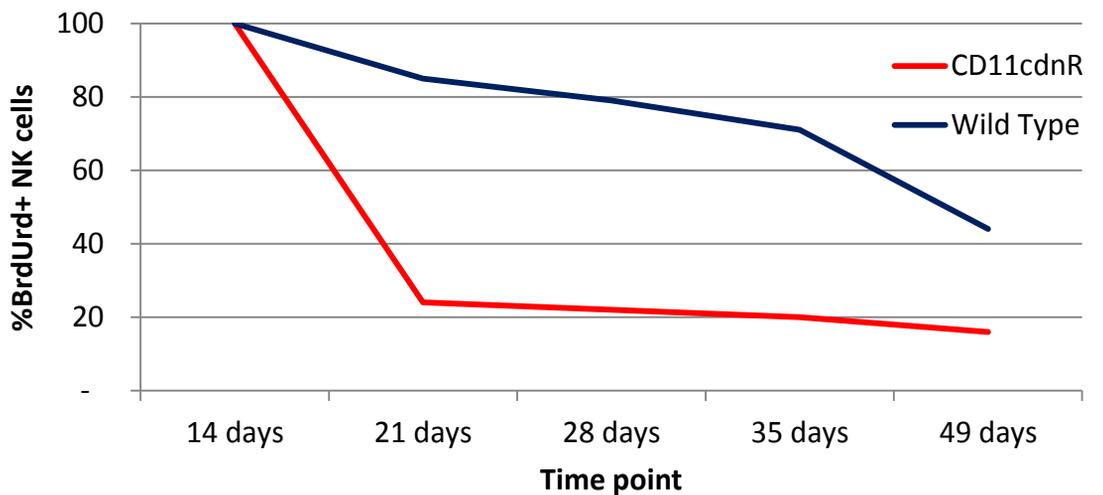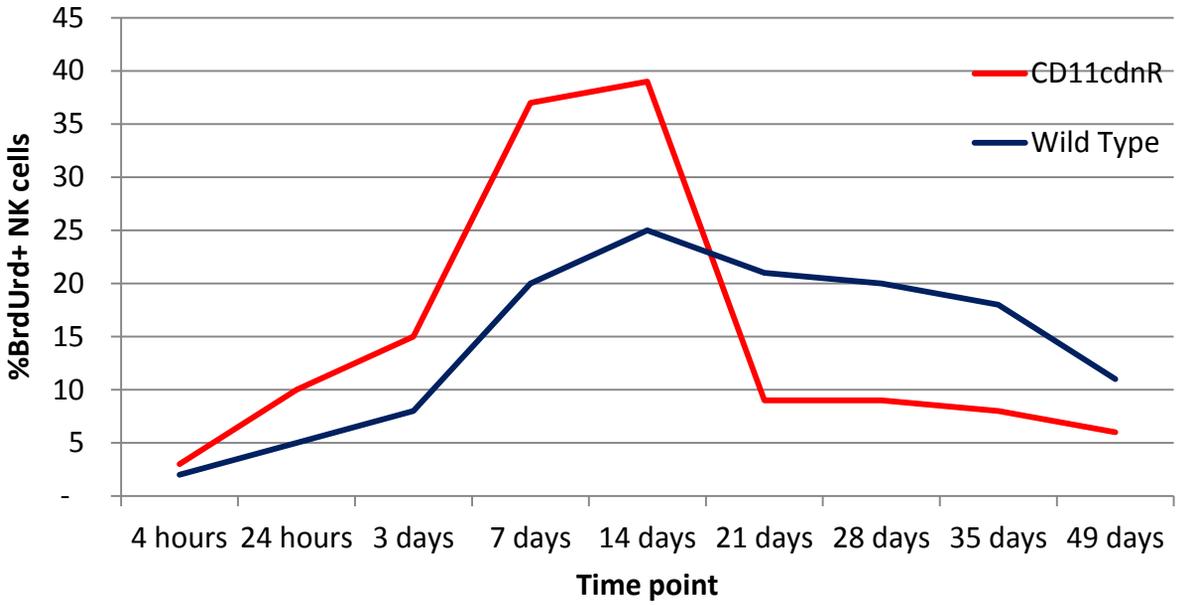
Figure 23 A

Figure 23 B

Figure 24 A Wild Type

Concentration IL-15

Gated on total splenic lymphocytes

Figure 24 B CD11c^{dnR}

Concentration IL-15

Gated on total splenic lymphocytes
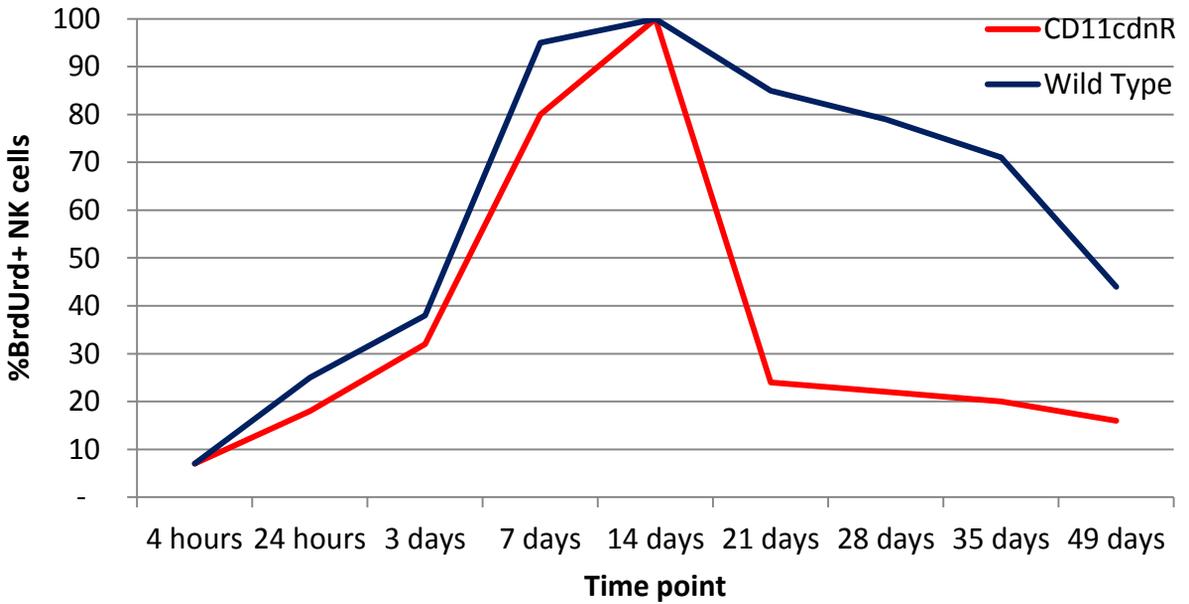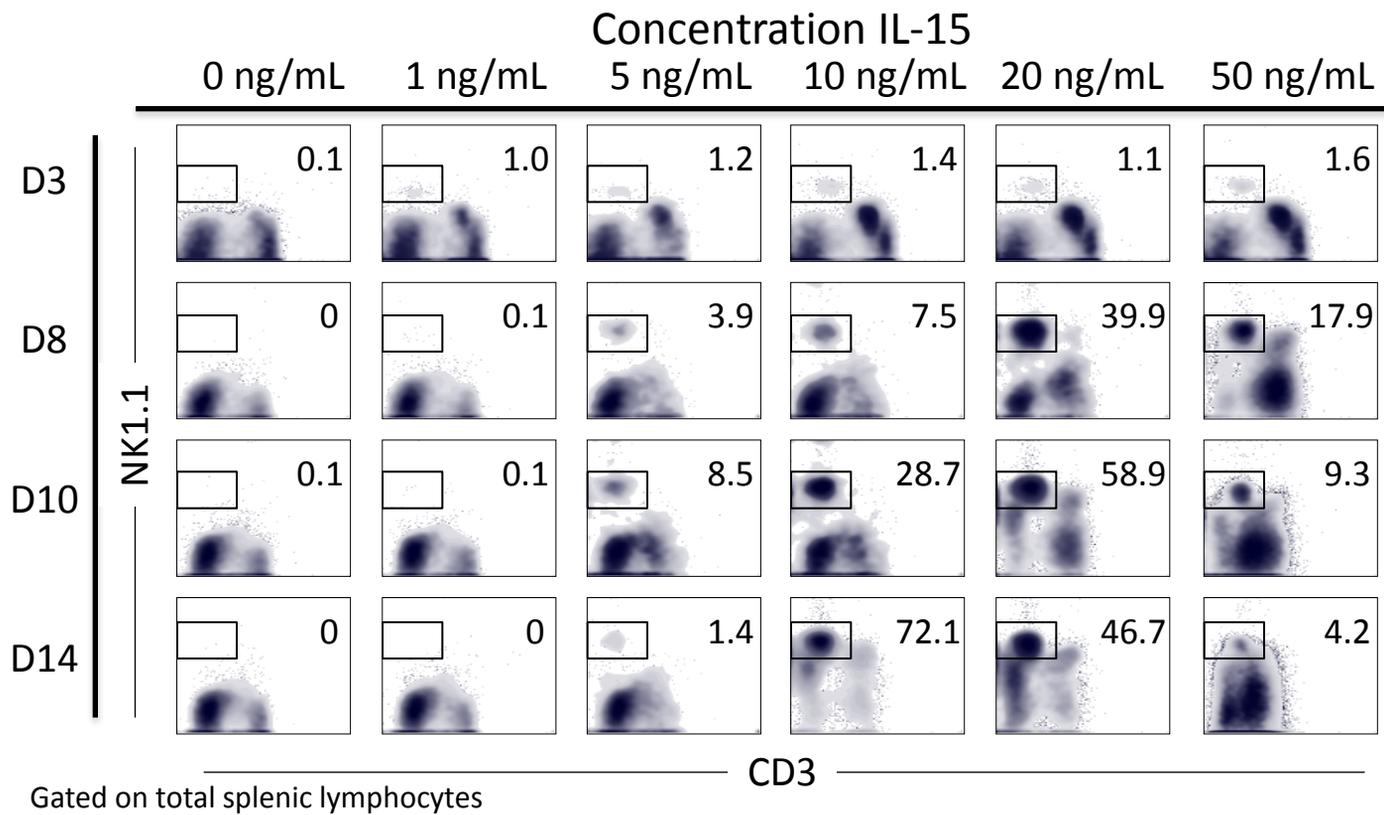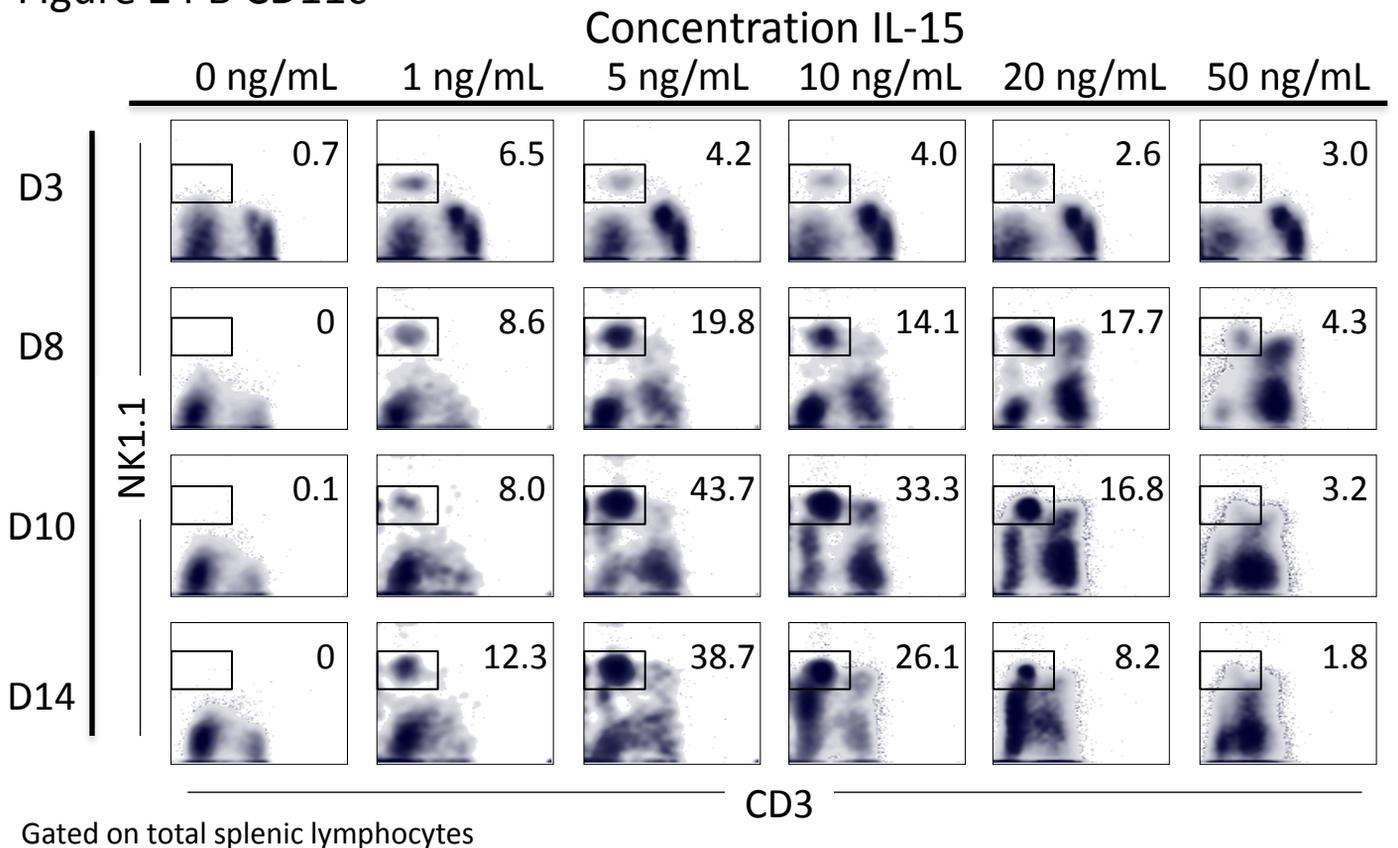
Figure 24 C Wild Type +5 ng/mL TGFβ

Concentration IL-15

Gated on total splenic lymphocytes
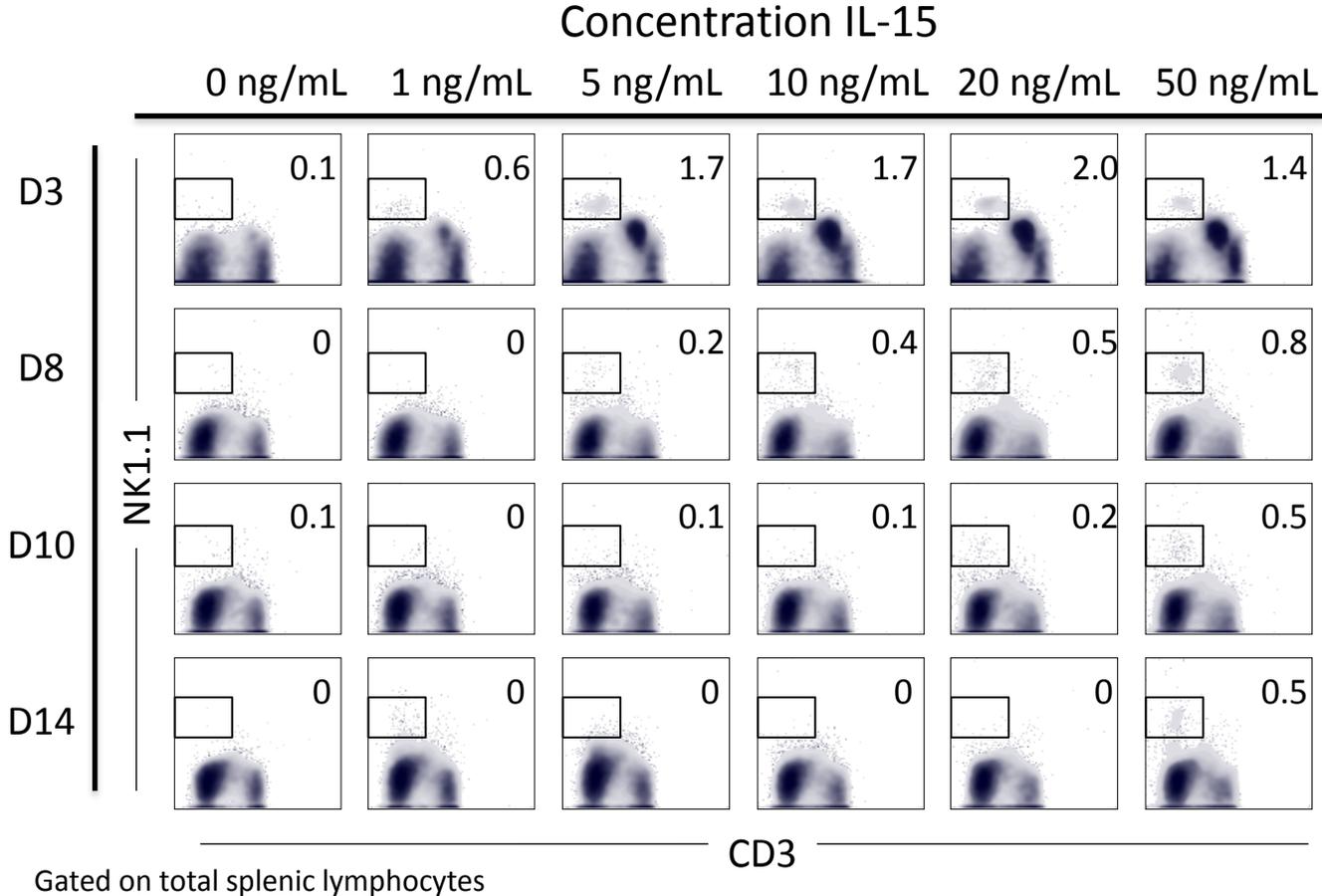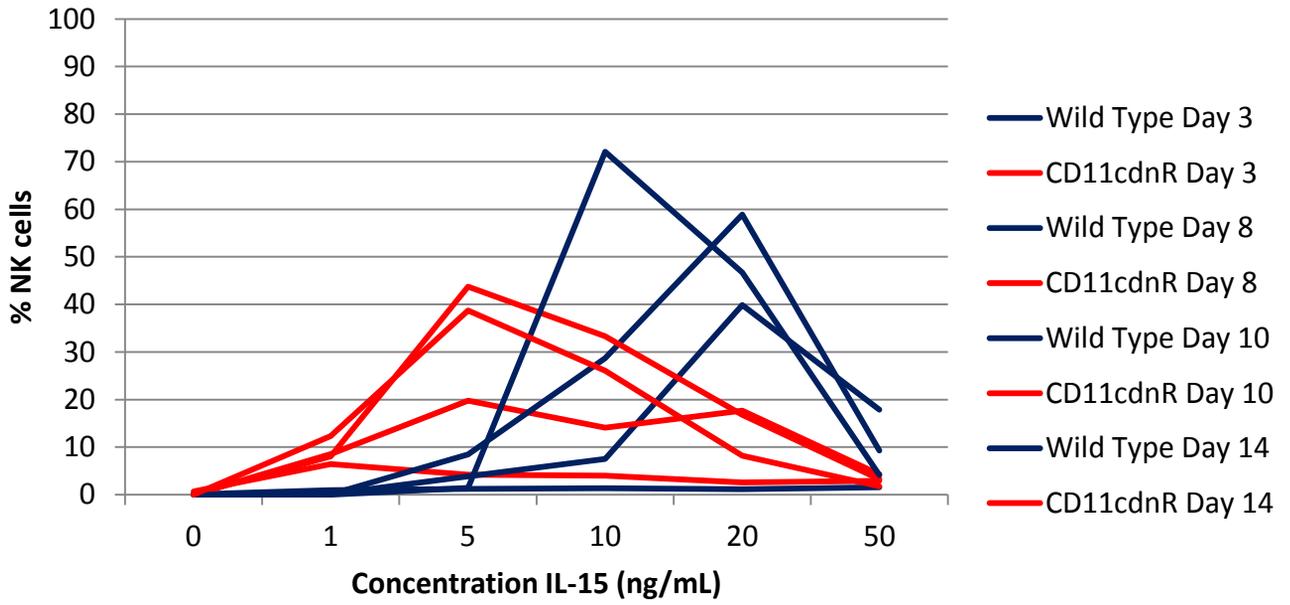
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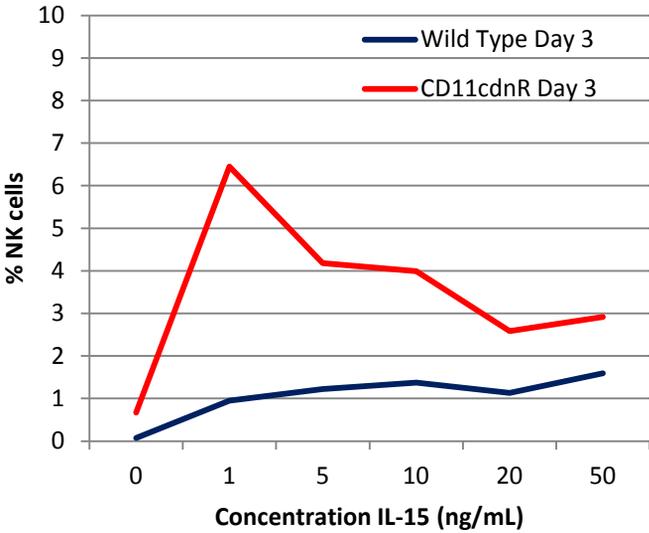
Figure 25 B

Figure 25 C
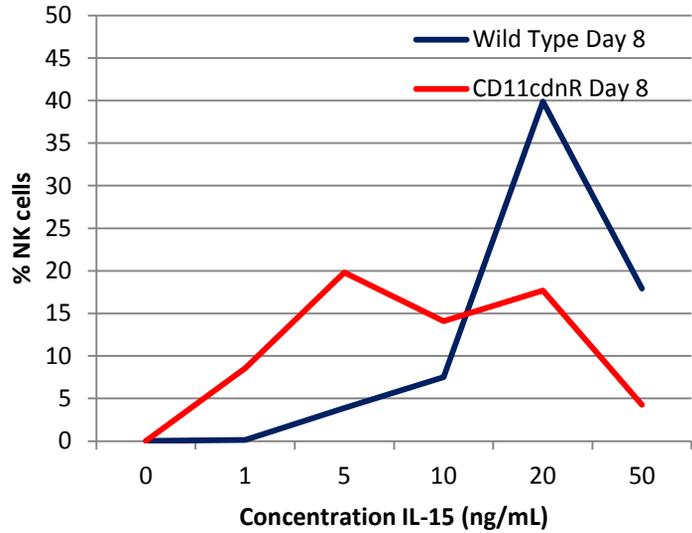
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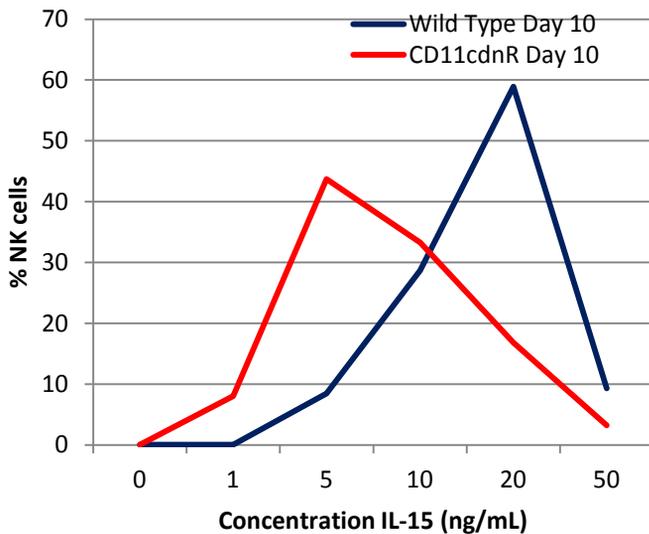
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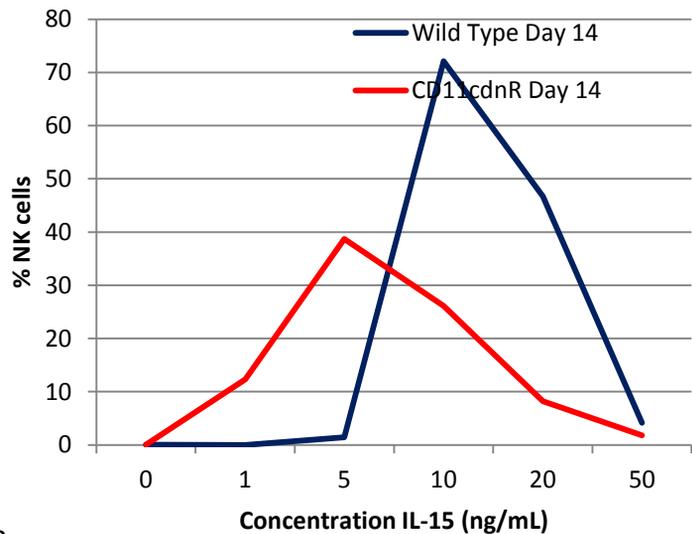
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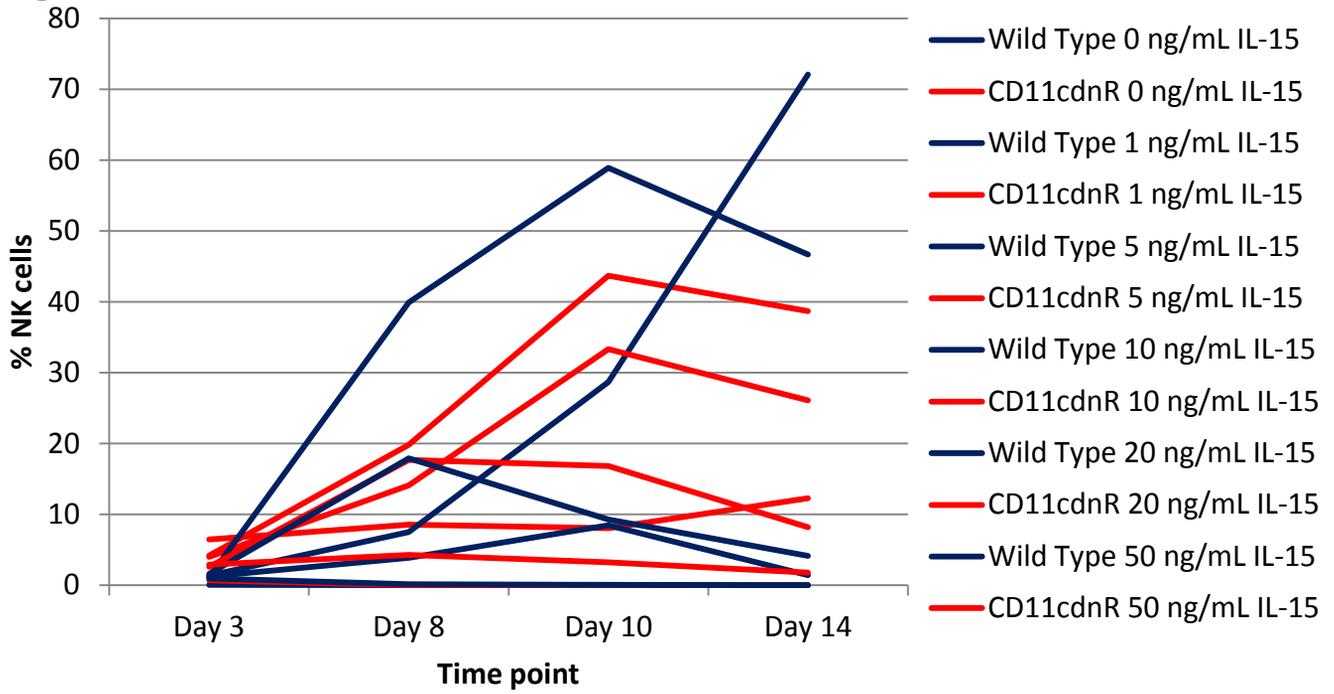
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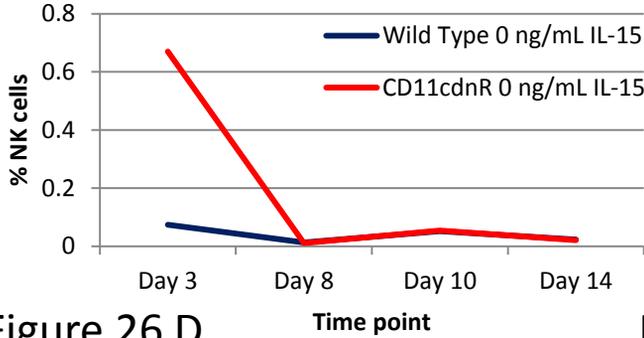
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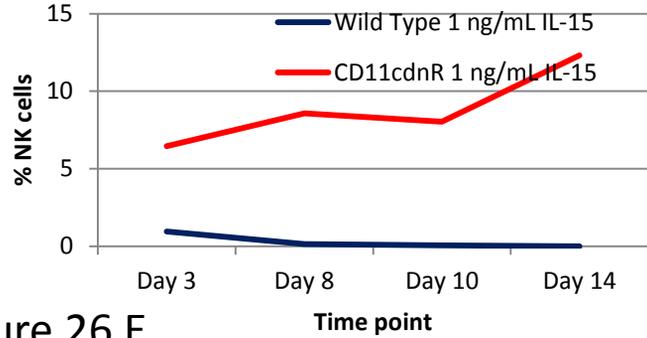
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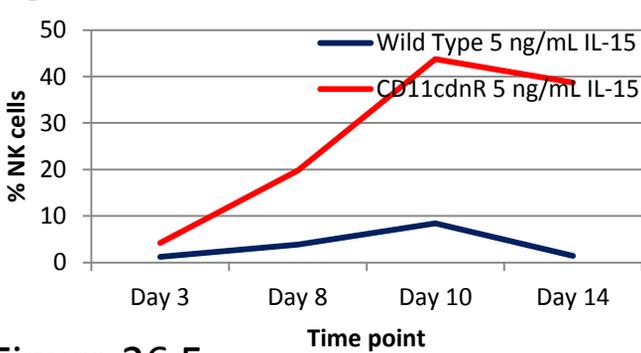
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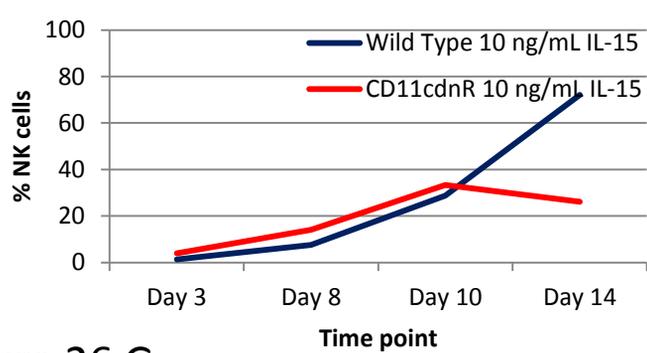
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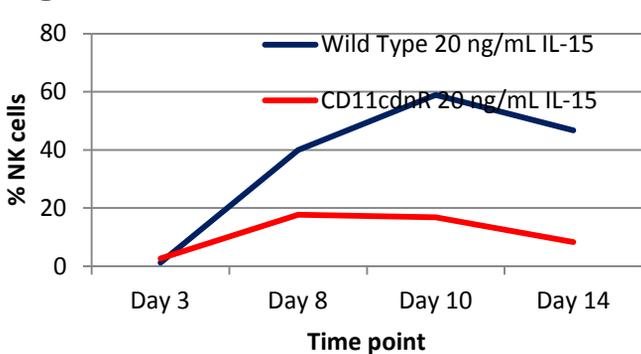
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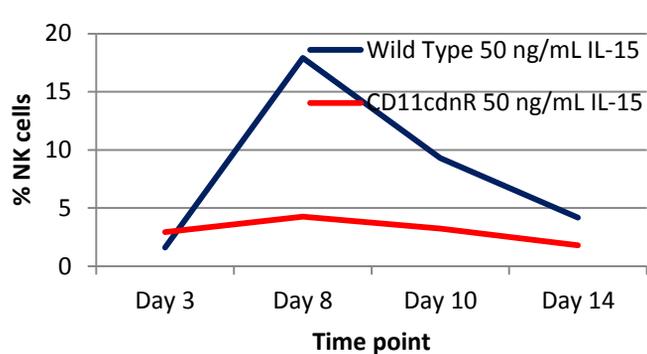
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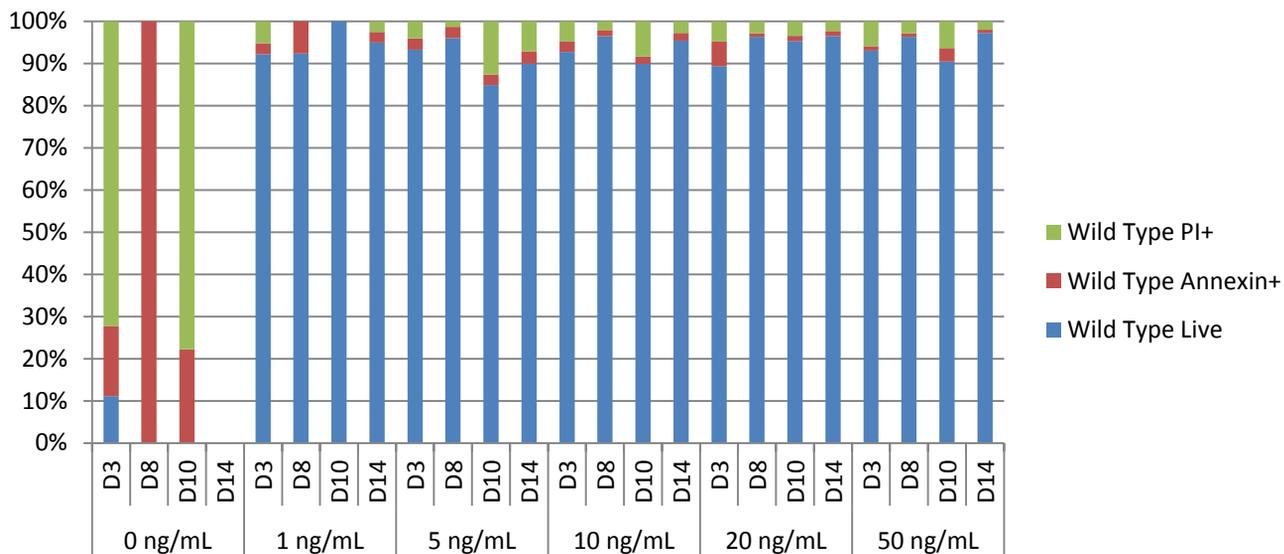
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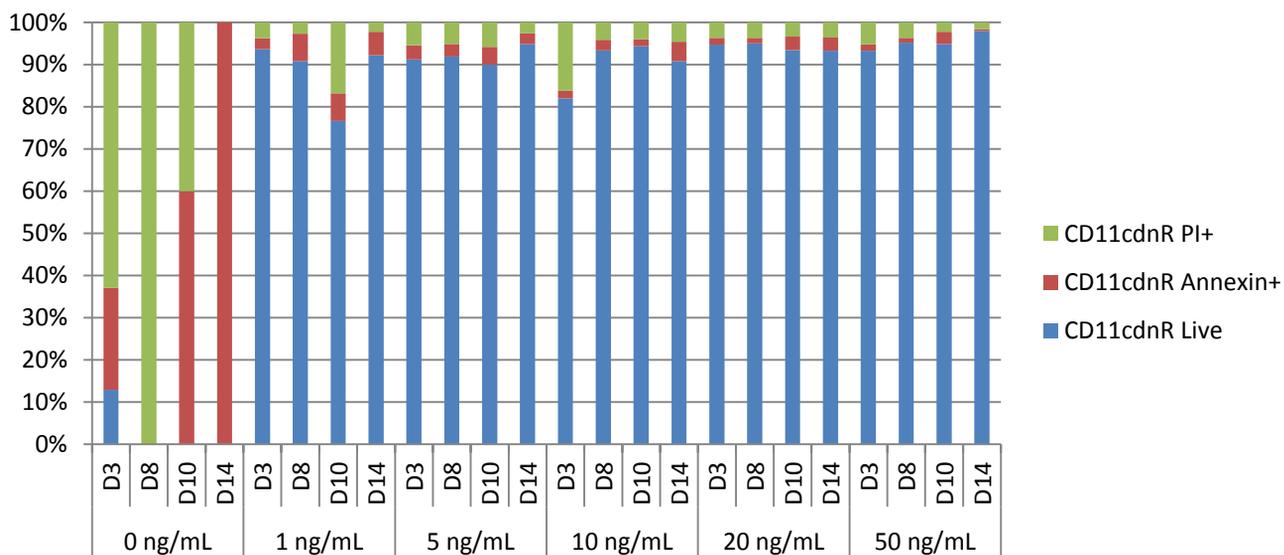
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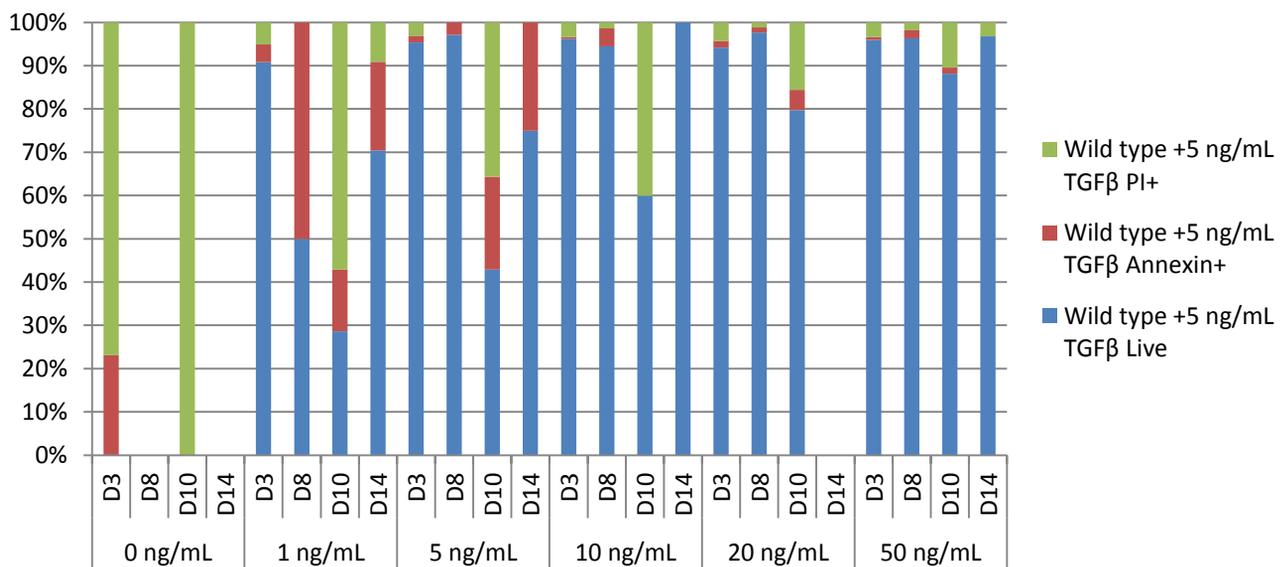
Figure 28 A

Concentration IL-15

Gated on total splenic lymphocytes
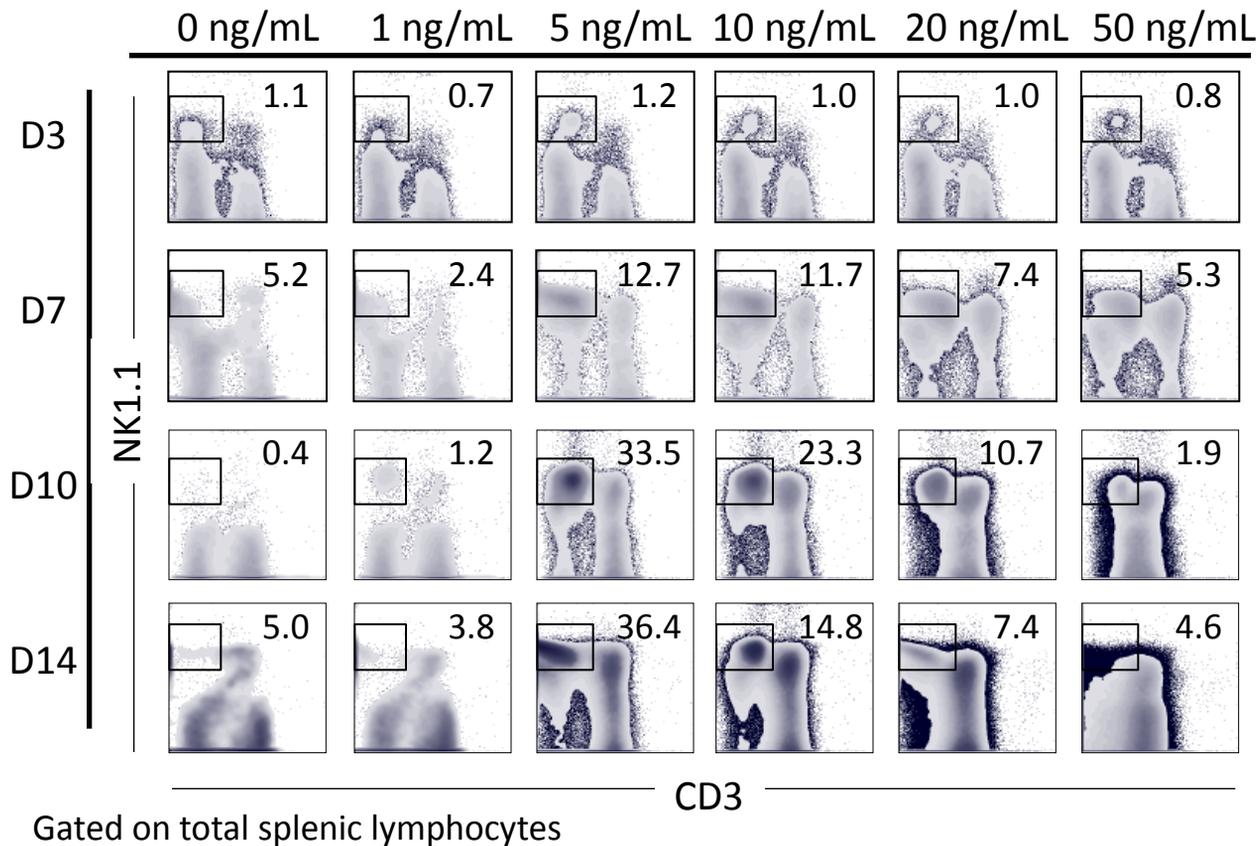
Figure 28 B

Concentration IL-15

Gated on CD3⁻NK1.1⁺DX5⁺ cells
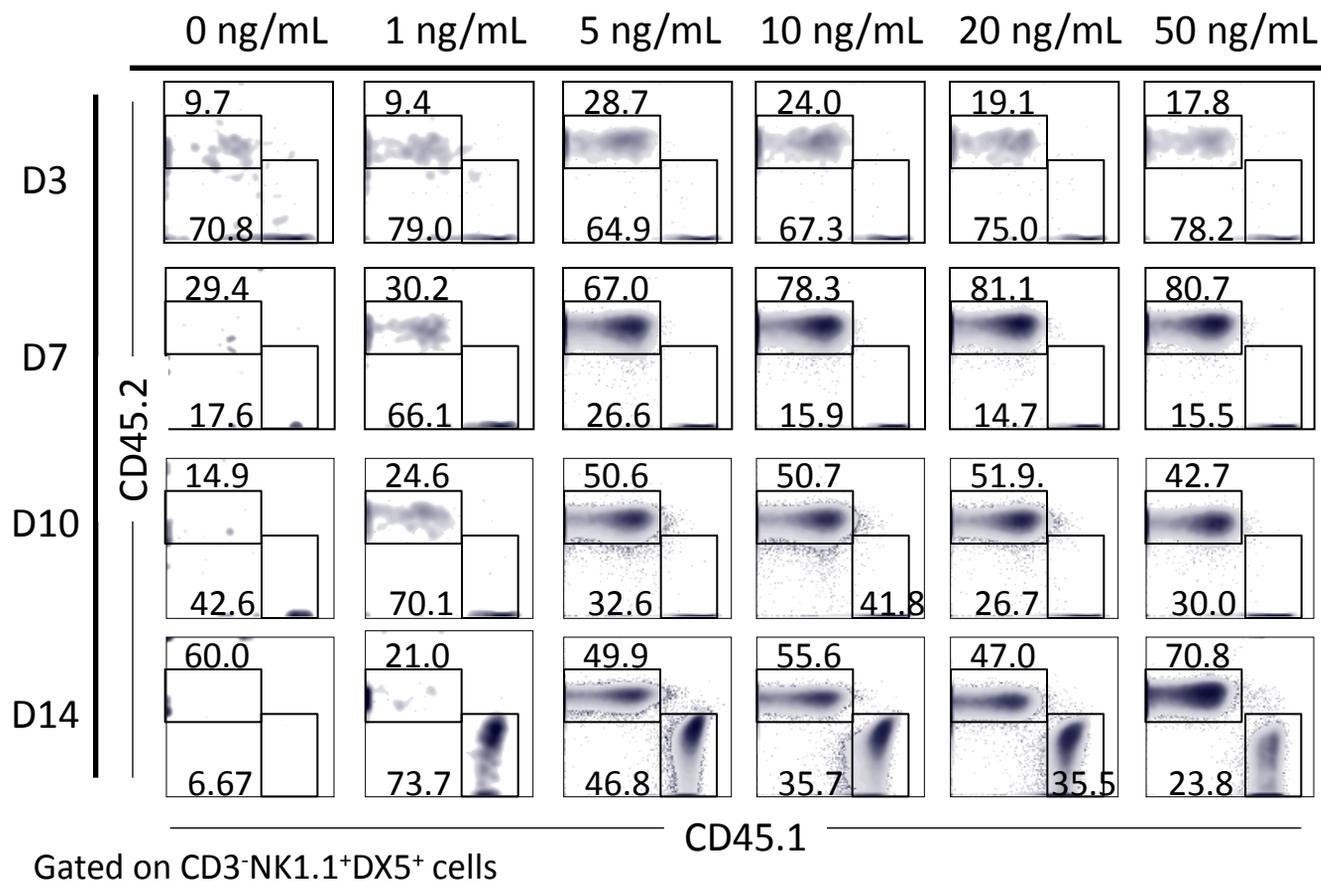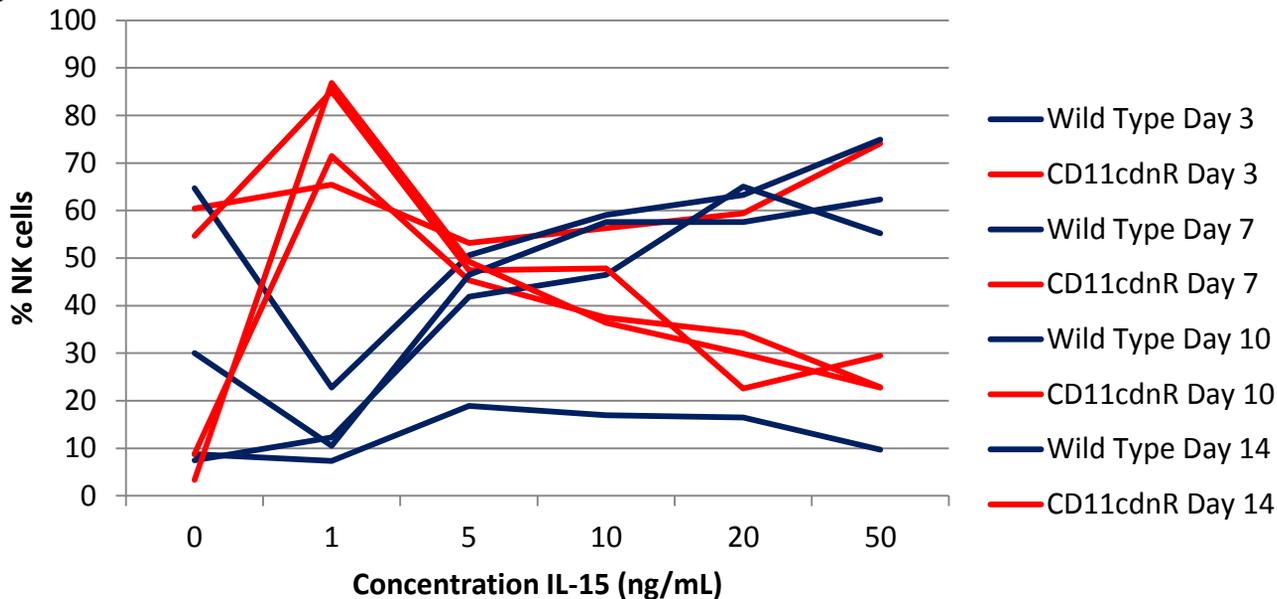
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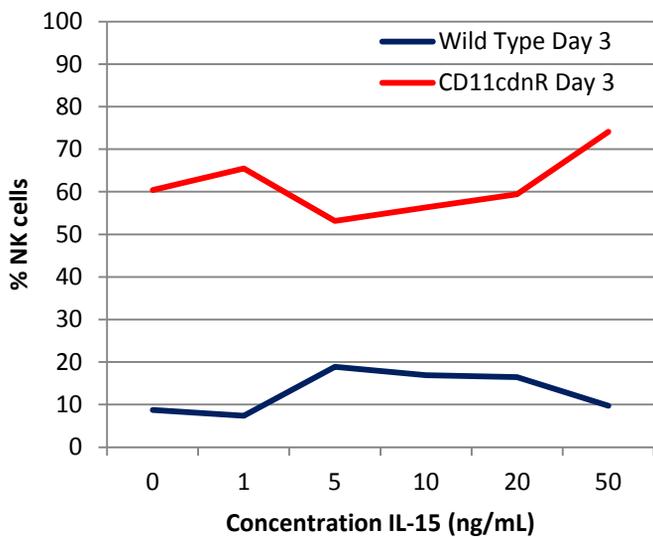
Figure 29 B

Figure 29 C
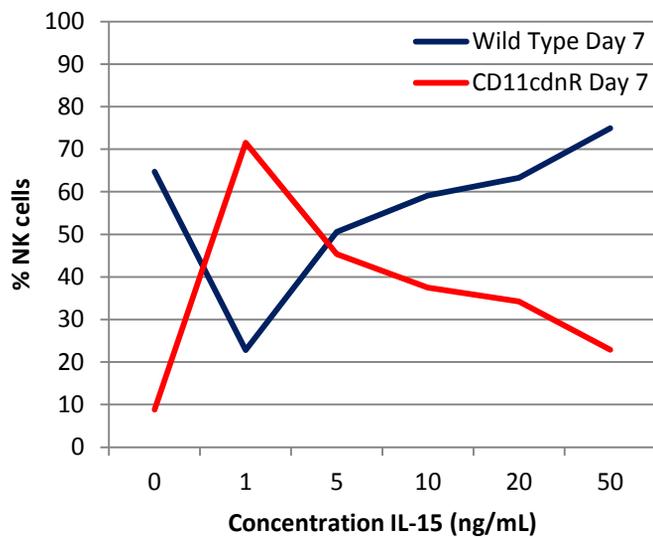
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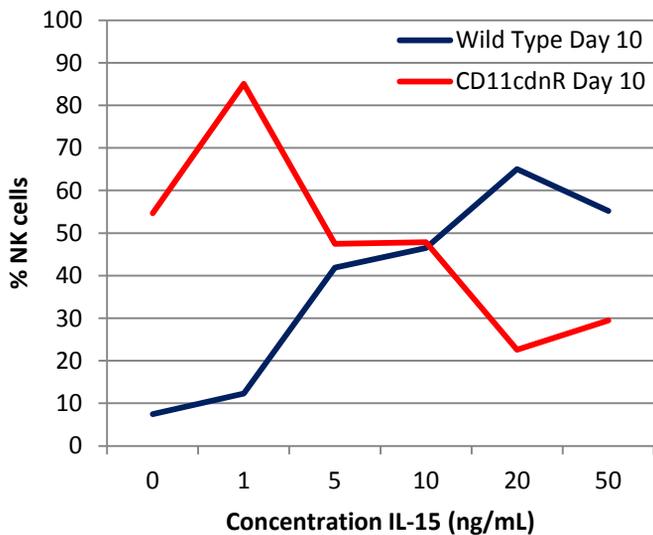
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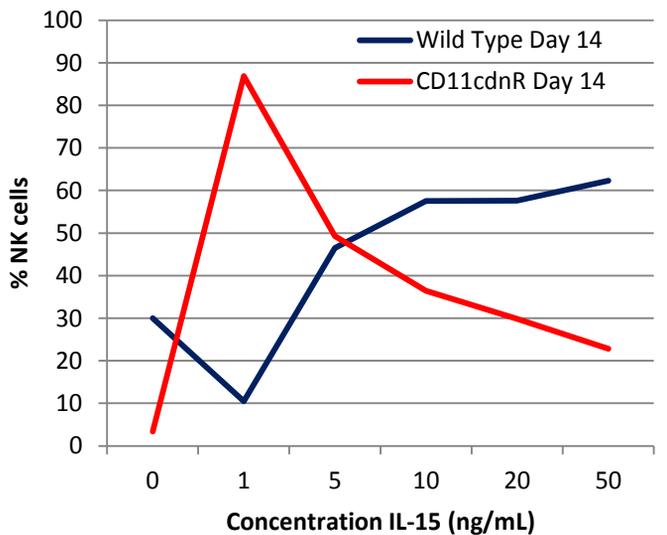
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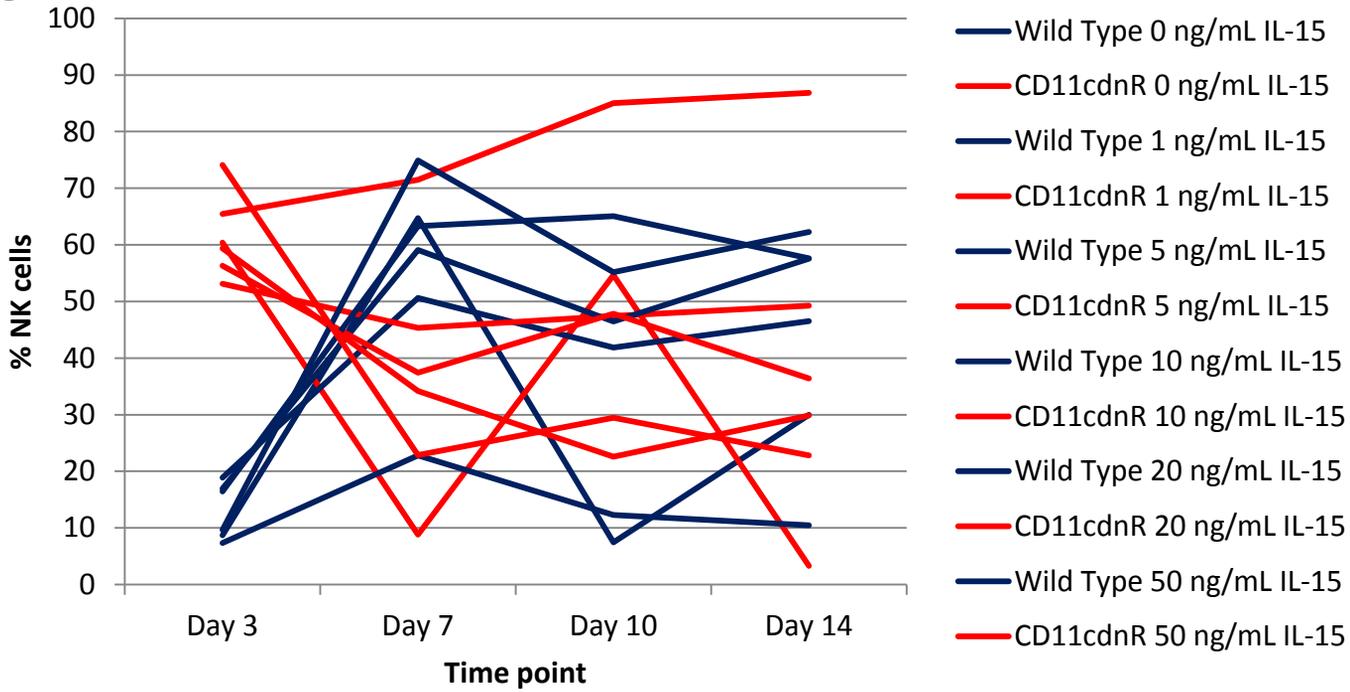
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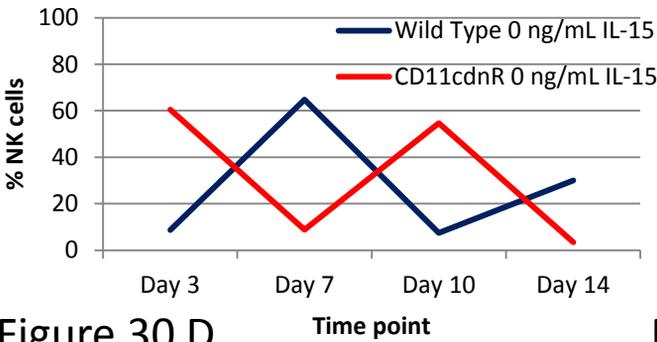
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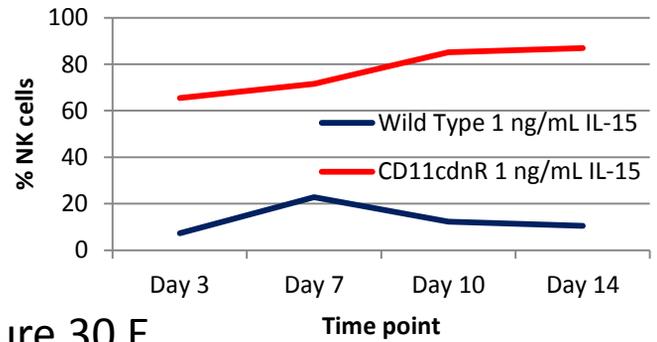
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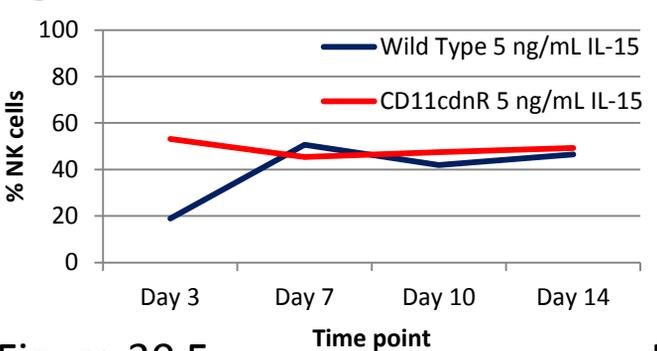
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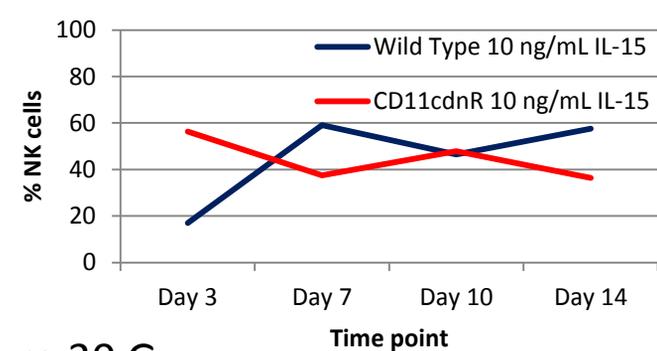
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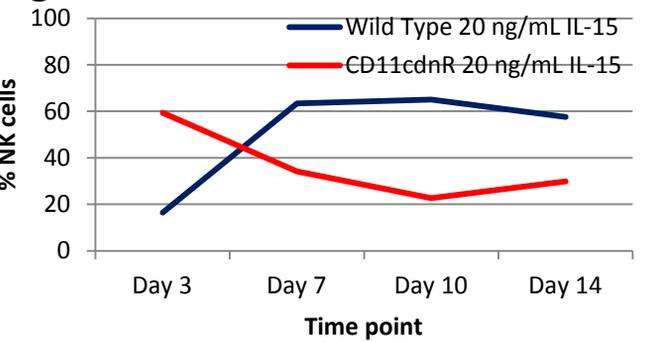
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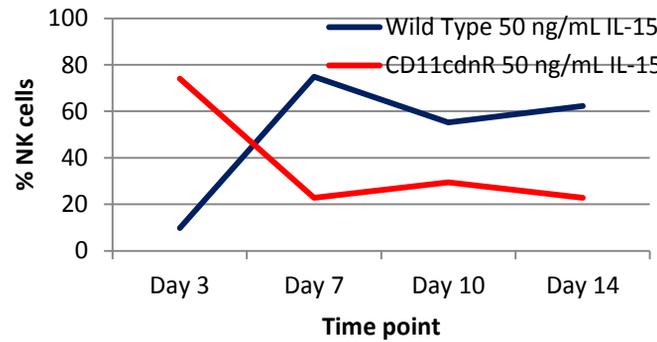
Figure 31 A

Figure 31 B

Figure 31 C