THE RAG RECOMBINASE DICTATES FUNCTIONAL HETEROGENEITY AND CELLULAR FITNESS IN NATURAL KILLER CELLS

by

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A Dissertation

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Date

Dedication

To my parents for their unwavering support and belief in me.

To my grandparents for their strength and dedication.

To my family for respect, laughter, and love.

"Science takes things apart to see how they work.
Religion puts things together to see what they mean."

- Lord Rabbi Jonathon Sacks, *The Great Partnership* -

Abstract

The emergence of the recombination activating genes (RAG) in jawed vertebrates endowed adaptive immune cells with the ability to assemble a diverse set of antigen receptor genes. In contrast, innate lymphocytes such as natural killer (NK) cells are not believed to require RAG, as normal NK cell numbers exist in RAG-deficient mice. Here, we report that select subsets of NK cells unable to express RAG or RAG endonuclease activity exhibit cell-intrinsic hyper-responsiveness and a reduced capacity to survive following virus-driven proliferation. This deficit in cell survival may stem in part from reduced expression of DNA damage response mediators and defects in the repair of DNA breaks. Evidence for this novel function of RAG was also observed in T cells and innate lymphoid cells (ILC). Our findings demonstrate that selective RAG expression during ontogeny produces functionally distinct subsets within the peripheral NK cell pool, and reveal an unexpected role for the RAG proteins beyond V(D)J recombination. We propose that DNA cleavage events mediated by RAG endow developing adaptive and innate lymphocytes with a cellular "fitness" that safeguards their persistence later in life during episodes of rapid proliferation or cellular stress.

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List of Abbreviations

AIDS: Acquired Immunodeficiency Syndrome

ADCC: Antibody-Dependent Cytotoxicity

ALL: Acute Lymphoblastic Leukemia

APC: Antigen Presenting Cell

ATM: Ataxia Telangiectasia-Mutated

ATR: Ataxia Telangiectasia and Rad3-related protein

B Cell: Bone lymphocyte (B cells mature in bursa of Fabricius in birds. Discovered in 1956 by Bruce Glick)

BCL-2: B-Cell Kymphoma 2

BCR: B Cell Receptor

BM: Bone Marrow

bZIP: Basic Leucine Zipper

C-terminal: Carboxy-terminal

CID: Combine Immunodeficiency

CD: Cluster of Differentiation

ChIP: Chromatin Immunoprecipitation

CHK2: Checkpoint Kinase 2

CLP: Common Lymphoid Progenitor

CTLs: "Cytotoxic" T Lymphocyte

DC: Dendritic Cell

DDR: DNA Damage Response

DNA: Deoxyribonucleic Acid

DNA-PK DNA-dependent Protein Kinase

DNA-PKcs: DNA-dependent Protein Kinase Catalytic Subunit

DSB: Double Strand Break

FLT-3: Fms-Like Tyrosine kinase 3

GFP: Green Fluorescent Protein

H2AX: H2A histone family, member X (becomes γ-H2AX when phosphorylated)

H3K4me3: trimethylated histone H3 lysine 4

HCMV: Human Cytomegalovirus

HIV: Human Immunodeficiency Virus

HMGB1: High Mobility Group Protein B1

HSC: Hematopoietic Stem Cells

Ig: Immunoglobulin

iNK: Immature NK cell

ITAM: Immunoreceptor Tyrosine-base Activation Motifs

ITIM: Immunoreceptor Tyrosine-based Inhibitory Motifs

IFNγ: Interferon-gamma

IL: Interleukin

i.p.: Intraperitoneal injection

i.v.: Intravenously injection

ILC: Innate Lymphoid Cell

IL2Rγ: Interleukin-2 Receptor subunit γ or "common gamma chain"

IR: Ionizing Radiation

KIR: Killer cell Immunoglobulin-like Receptor

KLRG1: Killer cell Lectin-like Receptor G1

KO: Knock Out (gene-deleted mice)

LCMV: Lymphocytic Choriomeningitis Virus

LN: Lymph Node

MCMV: Mouse Cytomegalovirus

MCM4: Mini-Chromosome Maintenance-deficient 4

MDSCs: Myeloid Derived Suppressor Cells

MHC: Major Histocompatibility Complex

mNK: Mature NK cell

MPEC: Memory Precursor Effector Cells

MP: Memory Precursor

MSC: Melanocyte Stem Cells

N-terminal: Amino-terminal

NHEJ: Non-Homologous End Joining

NF-kB: Nuclear Factor Kappa-light-chain-enhancer of activated B Cells

NK cell: Natural Killer cell

NKP: NK cell Progenitor

NKT: NK T cells

OVA: Ovalbumin

PAMPS: Pathogen-Associated Molecular Patterns

PC: Peritoneal Cavity

PCC: Post-Cleavage Complex

PHD: Plant Homeodomain

PI: Post Infection

PI3K: Phosphoinositide 3-kinase

PRRs: Pattern Recognition Receptors

RAG: Recombination Activating Genes

RFP: Red Florescent Protein

RORγt: Receptor-related Orphan Nuclear Receptor-γt

ROS: Reactive Oxygen Species

RSS: Recombination Signal Sequence

SCID: Severe Combined Immunodeficiency

SHP: Src Homology region 2 domain-containing Phosphatase-1

SLEC: Short-Lived Effector Cells

STAT: Signal Transducer and Activator of Transcription protein family

T cell: Thymus Lymphocyte

TE: Terminal Effector

TdT: Terminal Deoxynucleotidyl Transferase

TCR: T Cell Receptor

TGFβ : Transforming Growth Factor-β

V(D)J recombination: Variable to Distal to Joining regions of antigen receptor

VEX: Violet light-EXcited fluorescence protein

VSV: Vesicular Stomatitis Virus

WT: Wild-Type

YFP: Yellow Fluorescent Protein

CHAPTER 1: INTRODUCTION

I GENERAL PRINCIPLES OF THE IMMUNE SYSTEM

The immune system is a composted of a variety of specialized cell types and processes that protect the host from disease. To function properly, the immune system must detect a wide variety of foreign agents, known as pathogens, and distinguish them from the organism's own healthy tissue, or "self". Specialized hematopoietic stem cells (HSC) in the bone marrow undergo dedicated differentiation schemes to develop all cells of the immune system. Upon development, these cells migrate toward secondary lymphoid tissues (e.g. thymus, spleen, liver) for continued maturation or circulate in the blood or lymphatic system to detect potential threats to the body. In order to do so, all immune cells rely on a distinct set of receptors to help them discriminate "self" and healthy from "non-self" or diseased tissues. In jawed vertebrates, the immune system is classically assigned to either the innate or adaptive immune system. Historically, cells of the adaptive (or acquired) immune system create immunological memory after an initial response to a specific foreign particle, or antigen. This subset of long-lived cells can "remember" and produce an enhanced response to subsequent encounters with the same antigen. On the other hand, the members of the innate immune system are first responders to breach of protective barriers (e.g. skin). Interaction between the two systems is highly regulated and essential for the evolution of the immune system to recognize and neutralize pathogens.

1 Innate Immune system

Innate immune cells are the body's first line of defense against invasion since they can become activated without the need for prior sensitization. Cells of this nature are thought to be short-lived and do not confer life-long immunity (Medzhitov and Janeway, 2000). Upon penetration of physical barriers – epithelium or the mucosa – the cellular components of the innate immune system recognize general features commonly shared among pathogens (pathogen-associated molecular patterns (PAMPS)) with their germ-line encoded cognate pattern recognition receptors (PRRs). Upon detection, innate cells respond to pathogens within minutes to hours by producing pro-inflammatory cytokines and chemokines that lead to environmental changes at the site of infection and to mobilize other cells of the immune system.

Myeloid–derived neutrophils, eosinophils, basophils, macrophages/monocytes, mast cells, dendritic cells (DCs), and lymphoid-derived natural killer (NK) cells form the innate arm of the immune system. This arm also contains complement, which is a set of plasma proteins that get activated through a enzyme-cascade and assist in opsonizing pathogens for phagocytes, lyse affected cells, or produce inflammatory peptides (Tomlinson, 1993). Macrophages, neutrophils, and DCs are examples of innate cells that consume (or phagocytose) harmful pathogens, foreign particles (or antigens), and dead or dying cells in the bloodstream and tissues. Upon engulfment, some of these cells (e.g. macrophages and DCs) can also function as professional antigen presenting cells (APCs)

that display pathogenic peptides in the context of major histocompatibility complex (MHC) I or II on the cell surface for T cell recognition (Delves and Roitt, 2000). APCs also express co-stimulatory molecules (CD80 and CD86) and migrate to the lymph nodes, which is essential for peripheral T cell activation and mounting of an effective adaptive immune response (Delves and Roitt, 2000).

Thus, innate cells have a fundamental role in the initiation of adaptive immune responses, which demonstrates a vital interdependence among the innate and adaptive immune system in order to clear infectious agents efficiently.

2 Adaptive Immune system

In contrast to the innate immune system, cells of the adaptive immune system are highly specialized. Through a process of somatic gene recombination (Tonegawa, 1983), B (bone) and T (thymus) lymphocytes can generate an almost limitless number of different antigen receptors (T cell receptor (TCR) and B cell receptor (BCR)) from a small number of genes (Boehm, 2011). Maturing cells undergo regulated processes of selection (for unique receptors that will ultimately recognize foreign proteins or peptides) and deletion (of auto-reactive receptors; termed “clonal deletion” or “negative selection”) to produce a repertoire of cells expressing a single, unique, and functional receptor. These important processes maintain tolerance to self, whereas aberrant regulation can lead to autoimmunity and disease.

Due to the stochastic expression of receptors with a distinct specificity, only a small number of B or T cells within a host express the correct receptor for a given pathogen-derived protein or epitope. Therefore, upon antigen encounter, naïve B and T cells with the correct specificity take several days to weeks to differentiate and expand into effector cells. This process, termed "clonal proliferation", produces a multitude of antigen-specific cells from the very few B or T cells expressing the specific receptor for the encountered antigen. Thus, the nature of the adaptive immune system is highly specific, but slow, compared to the innate immune system.

For T cells, this activation is achieved through the recognition of processed peptides bound to MHC molecules on APCs in the lymph node. T cells come in two predominant flavors: $CD4^+$ T "helper" cells and $CD8^+$ "cytotoxic" T cells (CTLs). Helper T cells produce cytokines that stimulate the necessary immune cells to eradicate the foreign pathogen, while CTLs mediate direct cytotoxicity against cells presenting their cognate antigen through the release of lytic molecules. B cells, on the other hand, differentiate into either memory cells or antibody-secreting plasma cells upon recognition of their cognate antigen (whole protein, and not MHC-restricted). Secreted antibodies are a crucial part of the defense against extracellular pathogens and can induce antibody-dependent cytotoxicity (ADCC), opsonization of pathogens, or complement activation.

Once an infection is cleared, the bulk of the innate and adaptive cells die, however a small population of memory T and B cells will persist for the life of the host. This immunological memory provides a mechanism for cells that have previously encountered antigen to rapidly respond if the antigen is encountered again (Woodland and Kohlmeier, 2009). Immunological memory is not only critical for long-term protective immunity, but also is the fundamental principle behind the development of vaccines against viral pathogens.

Collectively, the rapidly responding cells of the innate immune system along with the highly specialized cells of the adaptive immune system operate in a complex and highly coordinated manner to control infection and cancer.

3 Natural Killer Cells: Bridging the gap between innate and adaptive immunity

Since their discovery 40 years ago, natural killer (NK) cells are thought to function alongside the innate immune system (Murphy, 2012) (Figure 1A). However, this classical view of NK cells has been rapidly changing in last decade. Recent evidence suggests that this cell type possesses traits attributable to adaptive immunity (Sun and Lanier, 2011; Vivier et al., 2011). These characteristics include education mechanisms to ensure self-tolerance during NK cell development (Orr and Lanier, 2010), and clonal-like expansion of antigen-specific NK cells during viral infection followed by the ability to generate long-lived progeny known as “memory” NK cells (Daniels et al., 2001; Dokun et al.,

2001; Sun et al., 2009, 2010) (Figure 1B). NK cell memory has also been described in a plethora of non-pathogen settings (Cooper and Yokoyama, 2010; Paust and von Andrian, 2011). Therefore, it is thought that these unique cells operate at the interface of the innate and adaptive immune systems.

II ANTIGEN RECEPTOR REARRANGEMENT

Lymphocyte antigen receptors on T and B cells are the means by which these cells sense the presence of antigens in their environment. An almost limitless number of antigens exist in the environment and amongst the millions of lymphocytes present in each of us - each cell expresses a distinct antigen receptor to combat this robust foreign antigen diversity. How is the genetic information for this limitless number of immune receptors encoded by our limited genome?

In 1955, Danish immunologist Niels Jerne postulated (Jerne, 1955) that every animal had the inherent capacity to produce a large amount of a diverse set of antibodies against antigens through a 'selective' process that occurred when the antigen transported a 'selected' globulin to plasma cells, which would make identical copies of the globulins presented to them. Jerne's natural selection theory, which suggested antigen selects a pre-existing antibody repertoire, won him the Nobel Prize in 1984. Frank Burnett expanded on and improved on Jerne's theory, arguing that antigen bound by specific receptors on surface lymphocytes instructed, rather than selected, cells to proliferate and differentiate

into clones that produce antibodies with antigen specificity (Burnet, 1976). Burnet's 'clonal selection theory' further states the expanded clone is responsible for the secondary response where the differentiated cells secrete antibody (Burnet, 1976). Burnet also proposed that potentially self-reactive lymphocytes are removed during development, a process now known as clonal deletion. Burnet won the Nobel Prize in 1960 for discovering immunological tolerance.

In 1965, Dreyer and Bennett hypothesized that the generation of antigen receptors occurred through somatic DNA rearrangement (Dreyer and Bennett, 1965), whereby the genome contains a large repertoire of antigen receptor gene and each of them encodes a separate, specific receptor (or antibody). However, at the time, it was widely believed that genomic DNA remained unchanged throughout ontogeny. In 1976, Susumu Tonegawa challenged this dogma by showing that through restriction enzyme analysis of genomic DNA from mouse embryos and a mouse cancer cell line, large pieces of DNA between two flanking regions were missing in the cancer cells, but not in mouse embryos (Hozumi and Tonegawa, 1976). This was the first genetic evidence that cells do undergo DNA rearrangement, which illuminated a mechanism by which antibody diversity could be achieved from a single gene (Hozumi and Tonegawa, 1976). Tonegawa provided the basic template for V(D)J recombination at antigen receptor loci and was awarded the Nobel Prize in 1987. While much progress has been made in understanding the basic mechanism of antigen receptor rearrangement, much is still unknown.

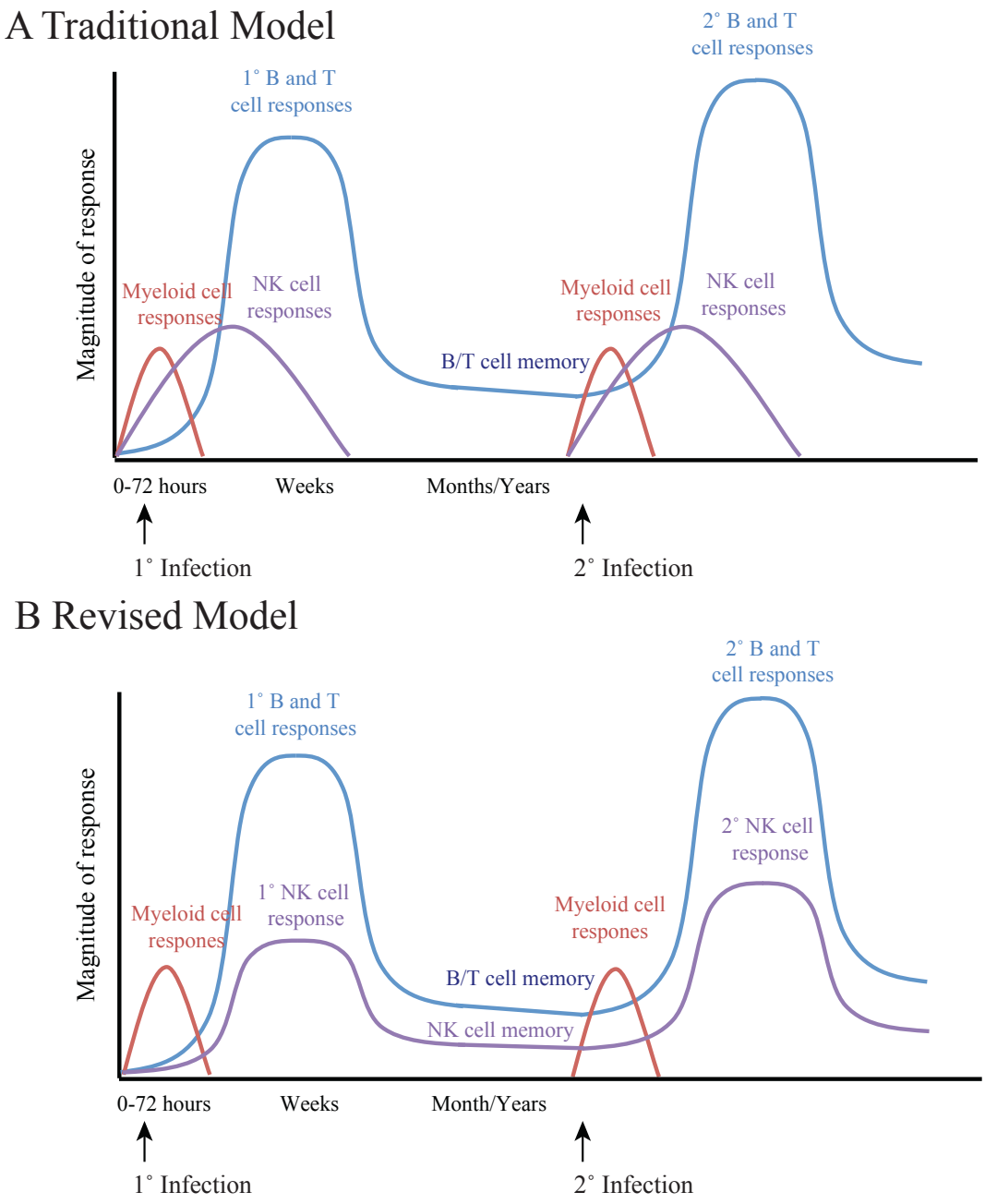


FIGURE 1 | TRADITIONAL AND REVISED MODELS OF INNATE AND ADAPTIVE IMMUNITY.

Graphs show the immune response against primary and secondary infections. The response of myeloid lineage cells (neutrophils, macrophages, and dendritic cells) is depicted by red lines. The NK cell response is shown in purple, and the response of B and T cells is shown in blue. The magnitude of the response refers to the qualitative nature of the immune response (i.e. efficacy (protective capability) and effector cytokine production) rather than the absolute number of cells.

1 Nuts & Bolts: Mechanism of Action

Ultimately, each lymphocyte expresses a unique surface receptor with specificity against a given foreign antigen. The diversity of these receptors is derived through extensive gene rearrangement. The process of V(D)J recombination is an intricate and tightly regulated process that requires the programmed induction and subsequent repair of DNA double strand breaks (DSBs) (Figure 2). DNA recognition and cleavage is mediated primarily by lymphoid-specific factors, while ubiquitous DNA repair pathways mediate end processing and joining. In the 1990s, it was shown that tightly timed expression of two genes, *Rag1* and *Rag2*, are responsible for the generation of antigen receptor diversity (Oettinger et al., 1990; Schatz et al., 1989). Further studies showed that these genes were evolutionarily conserved and transfer of a single genetic locus containing *Rag1* and *Rag2* (chromosome 11p in humans and 2p in mice) could make cells proficient at V(D)J recombination (Oettinger et al., 1992; Schatz and Baltimore, 1988; Schatz et al., 1989). In both humans and mice, point mutations that reduce or eliminate recombination activity of RAG1 and RAG2 lead to severe immunodeficiency due to the absence of antigen receptors and loss of lymphocyte development (Mombaerts et al., 1992; Schwarz et al., 1996; Shinkai et al., 1992).

Diversity in the antigen receptor repertoire is generated by shuffling of variable (V), diversity (D), and joining (J) gene segments that are found within the T cell receptor and B cell receptor loci (Bernard et al., 1978; Brack et al., 1978; Kurosawa et al., 1981; Weigert et al., 1970). After recombination, a completed receptor (TCR or BCR) is found

on the cell surface of T and B cells, or secreted receptor (antibody) from B cells. The germ-line consists of a multitude of V, D, and J gene segments, therefore random recombination results in the generation of approximately 10^6 different combinations (Nishana and Raghavan, 2012). RAG1 and RAG2 are thought to function as a heterodimer (collectively known as RAG), where RAG1 contains the endonuclease catalytic center (necessary for cleavage) (Kim et al., 1999; Landree et al., 1999), which is active in the presence of its binding partner RAG2 (Oettinger et al., 1990). More recently, the crystal structure of RAG was solved, and 2 molecules each of RAG1 and RAG2 were shown to form a 'Y-shaped' heterotetramer (230 kD), with the amino-terminal domains of two RAG1 chains forming an intertwined stalk (Kim et al., 2015). RAG proteins bind to highly conserved target recombination signal sequences (RSS) flanking the V, D, and J gene segments. These RSSs resides next to conserved segments containing a palindromic heptamer (CACAGTG) and an A/T-rich nonamer (ACAAAAACC) (Early et al., 1980; Max et al., 1979; Sakano et al., 1979; Schatz and Spanopoulou, 2005) separated by non-conserved spaces of either 12 or 23 bases (Figure 2). During V(D)J recombination only coding segments flanked by RSS with non-identical spacer lengths can be combined, known as the "12/23 rule" (van Gent et al., 1996). This spatial restriction helps prevent non-productive rearrangements.

RAG initiates recombination by nicking DNA at the border between the heptamer of RSS and the coding segment (Schlissel et al., 1993). The 3'-OH group at the coding end becomes covalently linked to the opposing phosphodiester bond resulting in a hairpin structure at the coding end and a blunt signal end (Roth et al., 1993). The broken DNA

ends remain associated with RAG proteins (Agrawal and Schatz, 1997; Hiom and Gellert, 1998) and ATM kinase (Bredemeyer et al., 2006; Helmink and Sleckman, 2012b) in a post-cleavage complex (PCC). The PCC is shuttled into the ubiquitous non-homologous end-joining (NHEJ) pathway for DNA repair (Agrawal and Schatz, 1997; Gellert, 2002; Lee et al., 2004) (Figure 2). RAG proteins are assisted by high mobility group protein B1 (HMGB1) and HMGB2 during cleavage (Aidinis et al., 1999), yet their defined role is still unclear (Schatz and Ji, 2011).

2 Fix-er-uppers: Non-Homologous End Joining Pathway

The introduction of DSBs by RAG leads to repair by the NHEJ pathway (Agrawal and Schatz, 1997; Gellert, 2002; Lee et al., 2004) (Figure 2). First, several PI3K-like Ser/Thr kinases are activated, including DNA-dependent protein kinase (DNA-PK) and ataxia-telangiectasia mutated (ATM) kinase, which orchestrate the DNA-damage response (DDR) (Nussenzweig and Nussenzweig, 2010). These kinases activate a multitude of downstream effector molecules that regulate transcriptional programs to balance pro-apoptotic (or death) and pro-survival pathways within rearranging cells (Bednarski and Sleckman, 2012). The Ku heterodimer, consisting of Ku70 (*Xrcc6*) and Ku80 (*Xrcc5*) binds to the broken DNA ends and forms a complex with DNA-PKcs (DNA-PK catalytic subunit) (Nussenzweig et al., 1996; Rooney et al., 2002). Artemis, an endonuclease, binds in conjunction with DNA-PKcs and helps resolve the hairpin formed during recombination (Ma et al., 2002). The processed ends are ligated by ligase IV/XRCC4 complex (Lieber, 2010; Rathmell and Chu, 1994). During this catalytic reaction, the

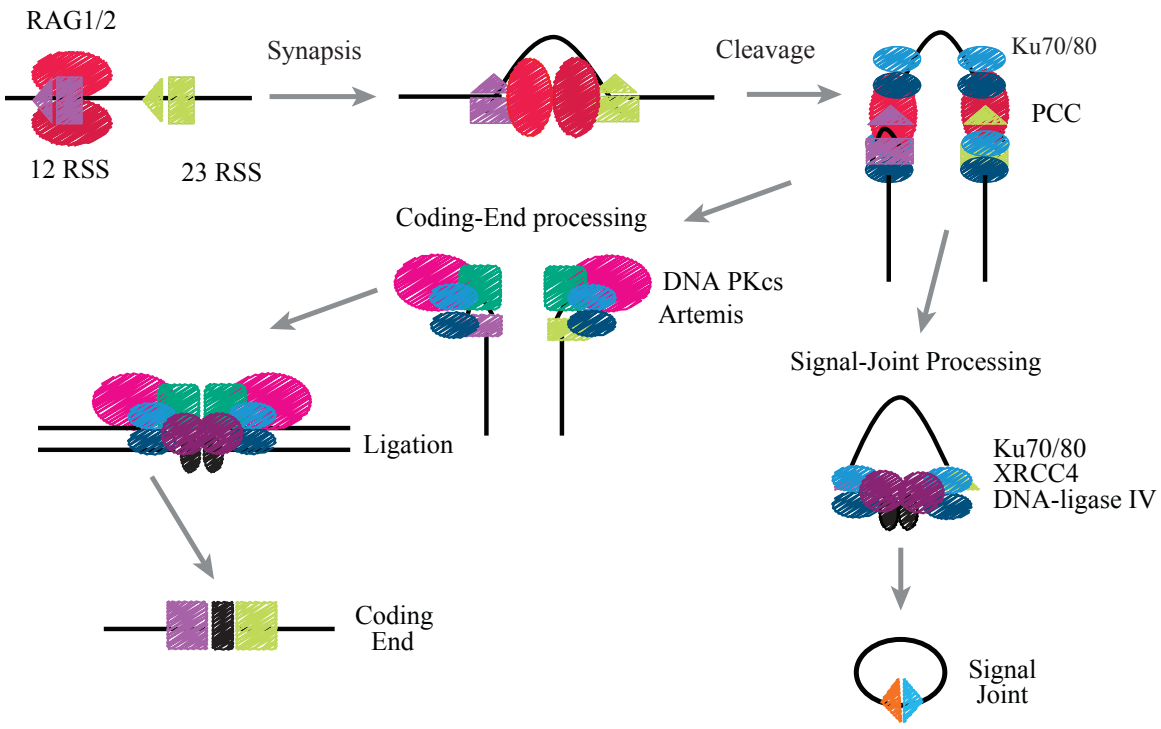


FIGURE 2 | OVERVIEW OF THE CLEAVAGE AND JOINING PHASES OF V(D)J RECOMBINATION.

Antigen receptor gene segments are flanked by a 12 recombination signal sequence (12RSS) or a 23 RSS. Cleavage phase begins with the binding of RAG1 and RAG2 to the 12RSS or 23RSS (12RSS depicted here), forming the 12 signal complex. Capture of the second RSS results in the formation of the paired complex, here RAG-mediated DSBs are formed. The RAG proteins hold the cleaved ends in a stable post-cleavage complex (PCC) and recruit non-homologous end joining (NHEJ) repair pathway members to ligate the broken DNA ends. Coding segment ends typically undergo nucleotide addition by TdT RSS ends are typically joined without processing to form the signal joint. Only one allele shown.

histone variant H2AX is phosphorylated (γ-H2AX) at the site of DSB formation, leading to the amplification of DDR and DNA repair (Fernandez-Capetillo et al., 2004; Rogakou et al., 2000b). To further increase receptor diversity, several insertions and deletions occur at the ligated junction by specialized proteins terminal deoxynucleotidyl transferase (TdT) and DNA Pol μ (Desiderio et al., 1984). This leads to a final antigen receptor count of approximately 10^{11} possible BCRs/antibodies for B cells and 10^{15} possible TCRs for T cells (Davis and Bjorkman, 1988; Nishana and Raghavan, 2012). High efficiency is required in this system, as aberrant rearrangement events can lead to apoptosis, genomic instability, and cellular transformation (Gladdy et al., 2003; Lieber et al., 2006; Mills et al., 2003).

3 The RAG Recombinase

The RAG recombinase is considered the tightly controlled gatekeeper to genetic diversity. Through biochemical analysis, the minimal region necessary and sufficient to perform V(D)J recombination, or "core" regions, of RAG1 and RAG2 were defined (Figure 3). Years after their discovery a 3.2A crystal structure of the heterotetramer was finally completed (Guma et al., 2006; Kim et al., 2015). The indispensible core region of RAG1 (amino acids 384-1008) is essential or all activities *in vitro* and *in vivo* (Sadofsky et al., 1993; Silver et al., 1993) including DNA cleavage, DNA binding, and RAG2 interaction. A triad of three acidic amino acids (D600, D708, and E962) (Fugmann et al., 2000b; Kim et al., 1999; Lin et al., 1999), known as the DDE motif, forms the catalytic center of the RAG complex. Further, the central core domain contains the RAG2 binding site and the C-terminal domain binds DNA (Arbuckle et al., 2001). The N-terminal non-

core region contains a RING domain, which exhibits ubiquitin ligase activity (Deng et al., 2015; Yurchenko et al., 2003). (Figure 3A).

Mouse RAG2 core (amino acids 1-383) forms a N-terminal 6-bladed beta-propeller domain (Callebaut and Mornon, 1998). The C-terminus contains a non-core plant homeodomain (PHD) finger that targets the complex to activated or "open" chromatin through binding of histone 3 trimethylated at lysine 4 (H3K4me3) (Ji et al., 2010; Liu et al., 2007; Matthews et al., 2007) (Figure 3B). H3K4me3 is known to be associated with transcriptionally active genes (Zhou et al., 2011). Therefore, recognition of the RSS and H3K4me3 chromatin mark by RAG is necessary to facilitate initiation of the recombination reaction. Recent genome wide CHIP-seq analysis indicates that RAG2 recruitment mirrors the footprint of this activation mark, binding to thousands of sites throughout the genome (Ji et al., 2010), whereas RAG1 binding is more directed and occurs predominantly at conserved RSSs (Figure 4). It remains to be determined if RAG1 binds outside the recombination center. Further, the C-terminus contains a threonine residue (T490) that acts as a target of CHK2 kinase (Lin and Desiderio, 1993) (Figure 3B). Phosphorylation of this amino acids regulates the proteosomal degradation of RAG2 at the G1/S transition of the cell cycle (Li et al., 1996), ensuring the precise temporal regulation of V(D)J recombination in lymphocytes during ontogeny.

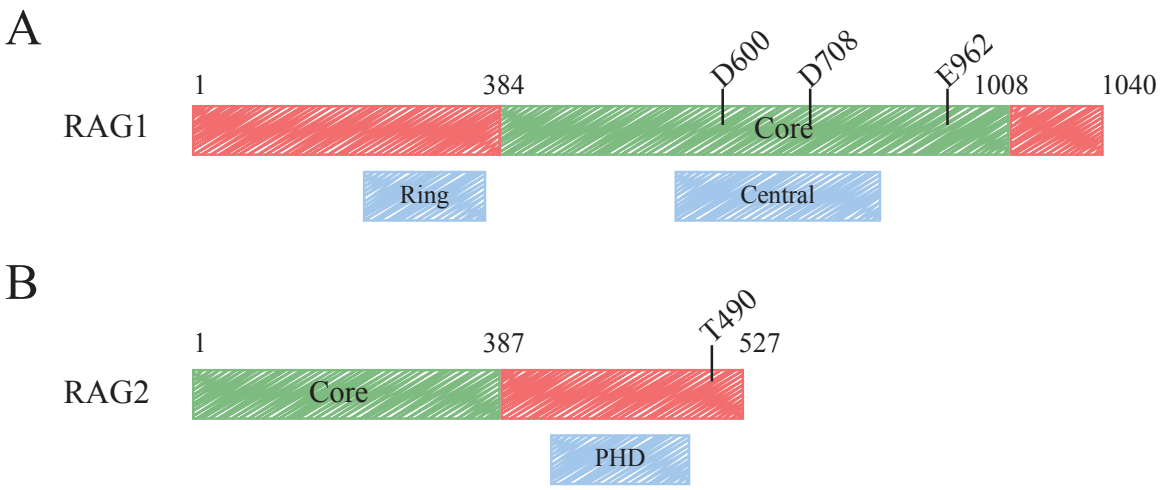


FIGURE 3 | STRUCTURE OF RAG1 AND RAG2.

Regions of RAG1 (A) and RAG2 (B) required "core" or dispensable "non-core" for basic recombination activity are shaded green or red, respectively. Key domains within these proteins and their location are shown below the full-length proteins (blue). Active site for DNA cleavage contains three acidic amino acids: D600, D708, and E962. RAG2 Carboxy-terminal region contains a PHD finger that binds H3K4me3 and phosphorylation site (T490) essential for its cell cycle regulation.

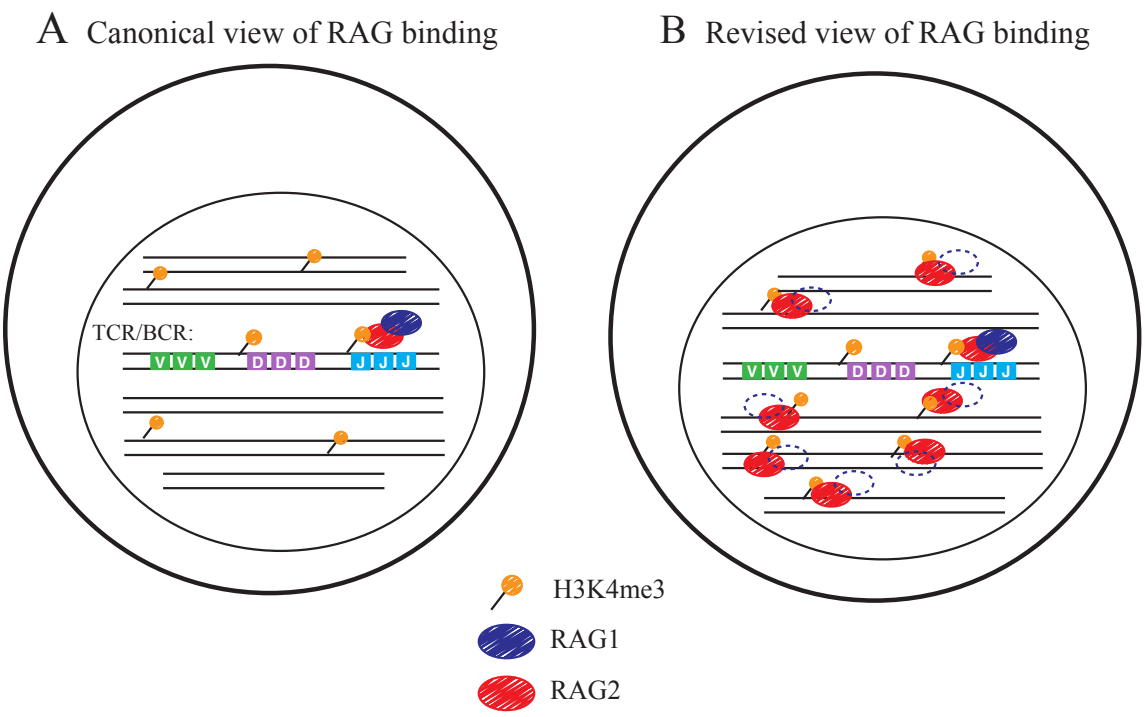


FIGURE 4 | CANONICAL AND REVISED VIEW OF RAG BINDING.

Traditionally, the RAG recombinase (RAG1/2) was thought to only bind to the antigen receptor loci (left panel) to facilitate rearrangement and generate receptor diversity in T and B cells. However, recent studies have led to a revision of the canonical view of RAG binding. In this revised view, RAG2 binds at points of 'active' chromatin throughout the genome (at thousands of sites), marked by the histone modification H3K4me3 (right panel). Whether RAG1 also binds outside the recombination center remains to be determined.

The RAG2 C-terminus domain is also required to escort the PCC into the canonical NHEJ pathway for proper DNA repair (Fugmann et al., 2000a; Lee et al., 2004). Genetic ablation of only the non-core domain in *Rag2*$^{c/c}$ mice (Liang et al., 2002) (which still contain a functional cleavage activity) has been shown to induce genome-wide chromosomal aberrations and lymphomagenesis when crossed to highly proliferative *p53*$^{-/-}$ mice (Deriano et al., 2011), suggesting a role for the non-core region in maintaining genomic integrity.

A Evolution of RAG

Based on core sequence homology, it is believed that RAG1 protein evolved from a transposon of the *Transib* superfamily (Kapitonov and Jurka, 2005), which is widely distributed in nematodes, insects, echinoderms, cniderians, and fungi (Kapitonov and Jurka, 2003). There is no consensus on when RAG1/2 became a permanent member of the vertebrate genome. Because agnathans ("no jaws") lack a core RAG1, it was traditionally assumed that RAG1 invaded after the agnathan/gnathatome (jawed vertebrate) split 1001 to 590 million years ago (Kasahara et al., 2004). Recently, the core sequence of RAG1 has been identified in the echinoderm *Strongylocentrotus purpuratus* (Purple sea urchin) (Fugmann et al., 2006), suggesting RAG1 may have invaded much earlier than originally thought. However, a RAG1 and RAG2 complex is only found in jawed vertebrates. The architectural similarity between RAG1 and the transposases Hermes (flies) and Tn5(bacteria) are further evidence for the evolutionary conservation of V(D)J rearrangement (Kim et al., 2015). It is hypothesized that this genome invasion is

the most important evolutionary event in immunology, leading to the formation of the adaptive immune system.

Recently, an evolutionarily conserved gene within the *Rag* locus, NWC, was found expressed in all cell types driven by its promoter, located within a *Rag2* intron (Cebrat et al., 2008). However, in lymphocytes, the canonical NWC promoter is silenced and transcription is placed under the control of the *Rag1* promoter (Laszkiewicz et al., 2012). It has been found that an antisense transcript generated by NWC transcription may negatively regulate *Rag* transcription (Laszkiewicz et al., 2014). It is speculated that the NWC locus may be the original site for RAG transposition.

4 Aberrant Translocations

Further studies have linked RAG to DNA breaks and chromosomal rearrangements (including translocations) at "cryptic" RSSs and non-RSS sequences that are scattered throughout the genome (Gostissa et al., 2011; Lieber et al., 2006; Mills et al., 2003; Papaemmanuil et al., 2014). In previously described oncogenic rearrangements, most translocational breakpoints on the non-antigen receptor gene partner contain RSS-like sequences at or near the breakpoint (Lewis et al., 1997; Marculescu et al., 2002; Raghavan et al., 2001; Tycko and Sklar, 1990; Zhang and Swanson, 2008). Specifically T cell acute lymphoblastic leukemia (T-ALL) and some pre-B ALL show evidence of translocations between cryptic RSS sites (Shimazaki and Lieber, 2014). Further,

recombination mediated malignancies often arise when DNA damage response machinery is compromised. For example, deleting NHEJ proteins in mice lacking p53 frequently leads to development of pro-B cell lymphoma initiated IgH-Myc translocation (Zhu et al., 2002). The function of RAG proteins at these cryptic RSS and transcription start sites remains to be determined.

5 RAG and Disease

Humans lacking RAG activity exhibit severe combined immunodeficiency (SCID) (Schwarz et al., 1996) with the absence of T and B cells but possessing NK cells, whereas partial loss of RAG activity results in Omenn syndrome (Villa et al., 1998) or combine immunodeficiency (CID). Mutations in non-core regions of both RAG1 and RAG2 cause Omenn syndrome or SCID in patients, indicating their importance in normal V(D)J recombination and lymphocyte development (Baker et al., 2008; Jones and Simkus, 2009; Villa et al., 2001). Recently, various mutations were mapped in a crystal structure of the RAG1-RAG2 heterotetramer (Kim et al, 2015). These mutations fall into four classes (1) destabilization of the tertiary structure, (2) areas likely necessary for DNA binding, (3) clustered around the active site, and (4) localized at the interface of RAG1 and RAG2 binding (Kim et al., 2015). Localization of these mutations reaffirm the importance of these functional and regulatory regions in V(D)J recombination.

III NATURAL KILLER CELLS

NK cells were initially described in 1975 as "natural killer" based on their ability to spontaneously kill tumor cells without pre-conditioning (Kiessling et al., 1975a; Kiessling et al., 1975b). As mentioned previously, these cells bridge the gap between the innate and adaptive immune system. Specifically, NK cells are specialized in recognizing aberrant cells in the body by integrating a balance of signals from activating and inhibitory receptors. Thus, NK cells act as a first line of defense before the development of adaptive immunity.

1 NK cell development

Many developmental stages occur in the bone marrow before an NK cell can become a full-fledged killer in the periphery. HSCs are functionally capable of self-renewal and generating all cell types in the blood, including lymphocyte and myeloid lineages, while the CLP has lost the ability to differentiate into myeloid or erythrocyte lineages. The CLP can give rise to T, B, and NK cell lineages in culture or upon transfer to recipient mice, and is defined as lineage (Lin)-negative (not committed to a mature cell type, CD3-CD19-Ter119-GR1-) and expressing cKit and IL-7Ra (Di Santo, 2006).

Commitment to the NK cell lineage requires the upregulation of CD122 the interleukin (IL)-2 receptor (Di Santo, 2006; Rosmaraki et al., 2001), through which NK cells receive

activation through IL-15 (Figure 5). NK cells are dependent on IL-15 signaling for development and survival in the periphery (Cooper et al., 2002; Di Santo, 2006; Prlic et al., 2003). These cells, known as NK cell precursors (NKP), have no B, T, myeloid, or erthyroid cell potential (Rosmaraki et al., 2001). Freshly isolated NKP are not cytotoxic for known mature NK cell targets. Furthermore, the commitment to the NK cell lineage is dependent on E protein transcription factors, Ets-1, and PU.1 (Barton et al., 1998; Boos et al., 2007; Di Santo, 2006; Yokota et al., 1999). Therefore, the process of generating NKP from HSC appears to be tightly controlled by transcription factors; however, the exact mechanisms by which they control NK cell development are unclear.

As NKP develop into immature NK (iNK) cells, they gain expression of several activating and inhibitory receptors, but lack the complete phenotypic and functional attributes of a mature NK (mNK) cell. iNK begin to upregulate Nkrp1c (NK1.1 in C57BL/6 mice – commonly used for identification) and Ly49 receptors (Lanier, 2005; Natarajan et al., 2002), and are found primarily in bone marrow and liver (Di Santo, 2006). Recently, the basic leucine zipper (bZIP) transcription factor E4BP4 (or NFIL3) has been shown to be essential for NK cell development from the NKP to iNK stage (Gascoyne et al., 2009; Kamizono et al., 2009). (Figure 5)

The conversion from iNK to mNK cell is distinguished by the expression of the integrin CD49b (DX5) and the capacity to kill and produce pro-inflammatory cytokines. The generation of mature peripheral NK cells appears to be dependent on the transcription

factors: GATA-3, T-bet, and IRF-2 (Lohoff et al., 2000; Samson et al., 2003; Townsend et al., 2004). mNK cells can generally be classified into subsets by their expression of CD27 and CD11b in mice (Kim et al., 2002). As immature, double negative cells mature, they gain CD27 expression. CD27+Cd11b- NK cells produce cytokines upon activation, but possess very little cytolytic capacity and are most concentrated in bone marrow, lymph nodes, and liver (Fu et al., 2011; Gregoire et al., 2007; Hayakawa and Smyth, 2006). Upon maturation, CD11b is expressed. Double positive, CD27+CD11b+ cells are distributed throughout the body and have the most pronounced production of cytokines and cytotoxic potential (Hayakawa and Smyth, 2006). The last stage of NK cell maturation is defined by loss of CD27 (Chiossone et al., 2009). These mNK cells are found in high concentrations within the spleen, blood, and lung (Hayakawa and Smyth, 2006). (Figure 5)

2 NK cell Receptors

NK cells do not rely on a single recombined receptor (e.g. TCR and BCR), but on a broad array of germ-line encoded activating and inhibitory receptors to distinguish host "self" from infectious "non-self" (Figure 6). Therefore, a balance of signals from various inhibitory and activating receptors determines the fate of the target cell that comes in contact with a potent natural killer.

A Inhibitory Receptors

Multiple classes of inhibitory receptors, which recognize MHC molecules on potential target cells, exist on NK cells (Natarajan et al., 2002) (Figure 6, red). Recognition of MHC class I or "self" on a target cell is an inhibitory signal to NK cells, protecting these cells from destruction. However, MHC is often downregulated by cells that are stressed, transformed, or virally infected. Therefore, the downregulation of "self" results in increased susceptibility to NK cell-mediated killing, a concept known as "missing self" (Ljunggren and Karre, 1990).

Ly49 receptors are a diverse set of type II C-type lectins that are expressed in mice encoded by a polygenic and polymorphic gene family *Klra* (Orr and Lanier, 2011). The family includes both inhibitory and activating receptors that bind to MHC class I-like proteins. In human, the functional counterpart of the Ly49 family is the killer cell immunoglobulin-like receptor (KIR) gene family, which encodes activating and inhibitory receptors that bind HLA class I molecules as ligands (Orr and Lanier, 2011).

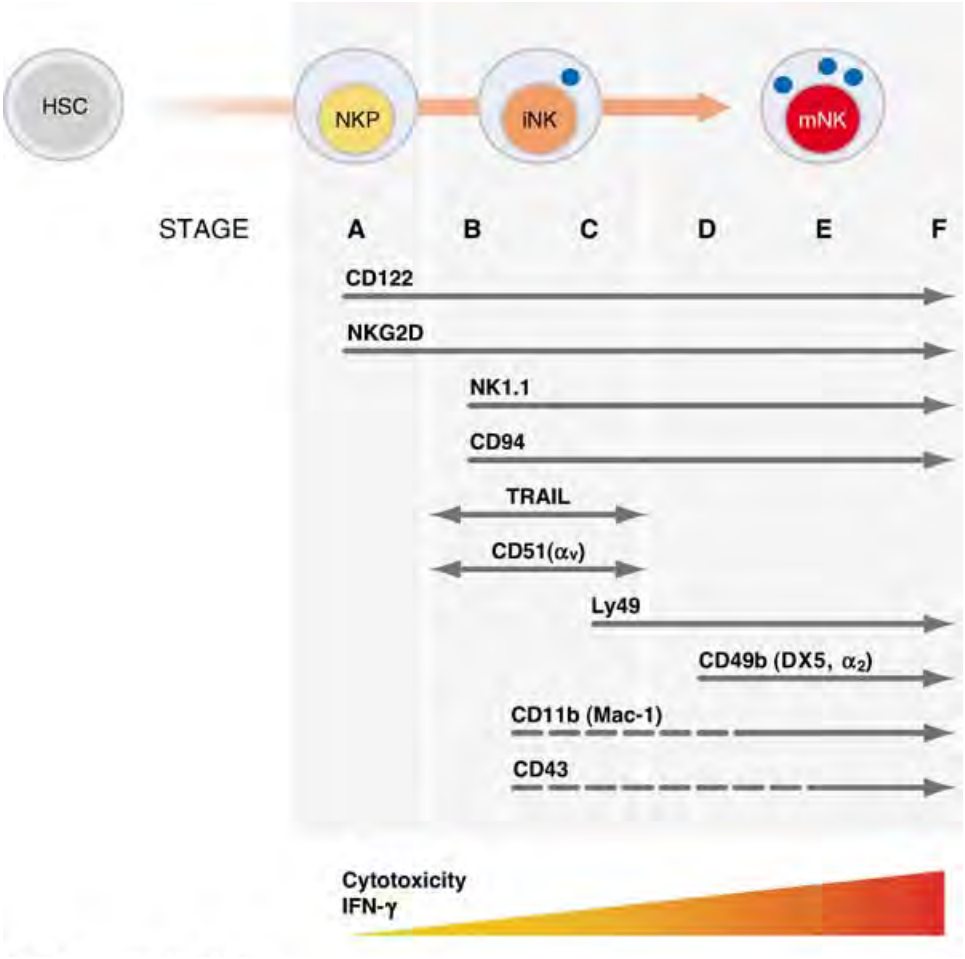
Upon interaction with MHC ligands, SHP-1 and SHP-2 tyrosine phosphatases are recruited to immunoreceptor tyrosine-based inhibitory motifs (ITIM) in the cytoplasmic domain of these receptors, inhibiting activation signals and NK cell activity. Most NK cells express at least one inhibitory receptor on their surface. Further, functional education, or "licensing", of NK cells is dependent on the interaction of an inhibitory

receptor with self-MHC molecules during ontogeny (Sun and Lanier, 2011). Inhibitory receptors work in concert with NK cell activating receptors to control NK cell reactivity.

Killer cell lectin-like receptor G1 (KLRG1) is an inhibitory receptor expressed on mNK cells, specifically NK cells undergoing proliferation or homeostatic expansion (Huntington et al., 2007). Generally, KLRG1+ NK cells are thought to be terminally differentiated and show poor expansion in adoptive transfer experiments post infection (Kamimura and Lanier, 2015). Memory NK cells express KLRG1 and have higher effector capacity than naïve NK cells (Bezman et al., 2012; Sun et al., 2009), similar to memory CD8+ T cells (Kaech et al., 2002).

B Activating Receptors

NK cell activation also requires signaling through activating receptors to trigger cytokine production and cytolytic activity (Lanier, 2008) (Figure 6, green). Signaling through activation receptors is coupled through DAP10, DAP12, and SAP/Fyn adaptors that contain immunoreceptor tyrosine-base activation motifs (ITAM) (Lanier, 2005). Upon phosphorylation of the ITAM, the tyrosine kinases Syk and ZAP70 are recruited via their SH2 domains and stimulate downstream events, including: Ca2+ influx, degranulation, and transcription of cytokine and chemokine genes (Lanier, 2005). Therefore, NK reactivity results from a delicate balance between activating and inhibitory receptors on NK cells.



Republished from: Di Santo JP. Natural killer cell developmental pathways: a question of balance. Annu Rev Immunol. 2006;24:257-86. PMID: 16551250.

FIGURE 5 | PHENOTYPIC MARKERS OF DEVELOPING NK CELLS IN THE MOUSE.

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.

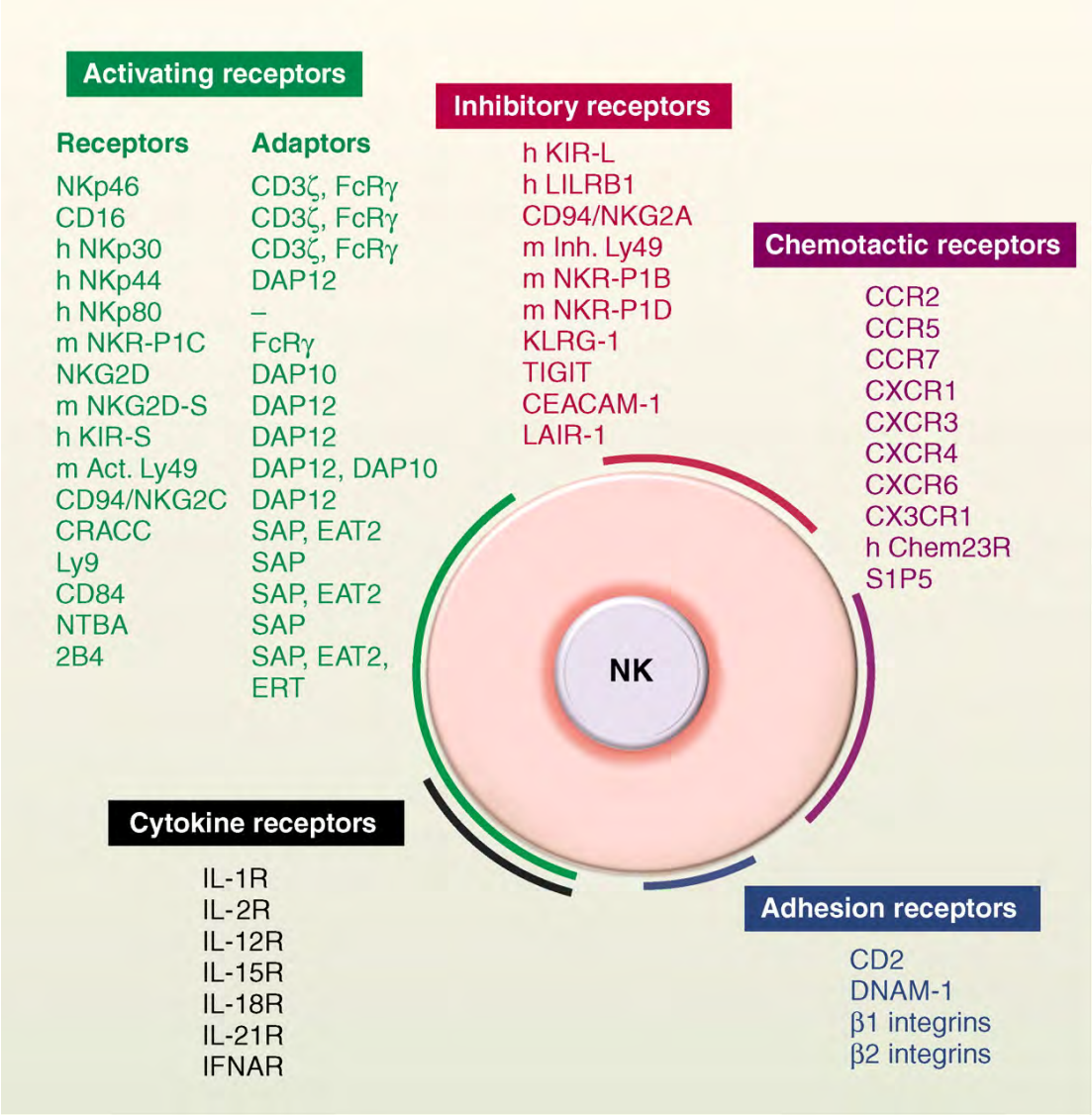


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FIGURE 6 | NK CELL RECEPTORS.

NK cells express many cell surface receptors that can be grouped into activating (green), inhibitory (red), adhesion (blue), cytokine (black) and chemotactic receptors (purple). In addition to MHC class I–specific receptors, other NK cell inhibitory receptors specific for non-MHC ligands also regulate NK cell reactivity. Adaptor molecules involved in the signaling cascade downstream of the engagement of activating receptors (green) are also indicated. The list of cell surface molecules involved in the regulation of mouse and human NK cell function is not exhaustive. Unless indicated (h, human; m, mouse), receptors are conserved in both species.

3 NK cell effector function

The integration of signals from activating and inhibitor receptors is decisive for the NK cell to trigger an effector function. Upon activation, NK cells release lytic molecules (e.g. perforin or granzyme) or membrane-bound death receptors that ultimately kill the target cell. Perforin forms pores in the target cell membrane, which disrupts the membrane integrity and allows the entry of granzymes (Hoves et al., 2010; Lieberman, 2003). Granzymes are a family of serine-proteases that induce caspases, which trigger the apoptotic (or cell death) program in the target cell (Lieberman, 2003). The importance of perforin was shown by perforin-deficient mice, which were unable to control transplanted tumor cells (van den Broek et al., 1996). Cell-surface bound CD107α (LAMP-1) protects NK cells from their own granzyme release (Cohnen et al., 2013).

In addition to a cytotoxic response, NK cells also release various cytokines that enhance and coordinate the immune response. Interferon-gamma (IFNγ) is considered to be the signature cytokine produced by NK cells. While this is a pleiotropic cytokine, its main functions include: activation of innate cells (e.g. macrophages and DCs), and promotion of CTL maturation, and various affects on tumor cells (Dunn et al., 2006).

4 Pathological Functions of NK cells

A NK cells and infectious disease

In humans and mice, NK cells play an important role in the control of infectious disease (Biron et al., 1989; Etzioni et al., 2005; Orange, 2006). Humans specifically lacking NK cells or NK cell effector function are particularly susceptible to herpesvirus family members, including human cytomegalovirus (HCMV), Epstein-Barr virus, and varicella zoster (Biron et al., 1989; Etzioni et al., 2005; Orange, 2006). Although HCMV infects a majority of the human population, it does not cause any life-threatening disease in healthy individuals. Studies suggest constant immunosurveillance in these individuals by NK cells at early stages of infection keeps HCMV in a latent state. In newborns and immunosuppressed individuals (including AIDS patients, cancer patients undergoing radiation therapy, and transplant patients treated with immunosuppressive drugs), HCMV infection can cause life-threatening complications (Biron et al., 1989; Etzioni et al., 2005; Orange, 2006).

NK cells have been shown to play a central role in immune-mediated control of mouse cytomegalovirus (MCMV) infection (Bukowski et al., 1985; Scalzo et al., 1990; Tay and Welsh, 1997; Welsh et al., 1991). Depletion of NK cells in resistant mouse strains using monoclonal antibodies (α-NK1.1) resulted in a significant increase in viral burden

following MCMV challenge (Brown et al., 2001; Bukowski et al., 1983; Daniels et al., 2001). Similarly, mice with genetic deficiencies in NK cell effector function (Bancroft et al., 1981; Shellam et al., 1981), as well as neonatal mice that lack mature NK cells, are more susceptible to MCMV infection compared to controls (Bukowski et al., 1985). A greater understanding of the molecular mechanisms behind NK cell activation and control during CMV infection and other infectious diseases is necessary for development of improved therapies against these pathogens.

B Role of NK cells in Tumorigenesis

NK cells were first identified by their ability to kill transformed cells without previous sensitization (Raulet and Guerra, 2009; Wu and Lanier, 2003). Thus, NK cells represent a promising immunotherapy for the treatment of cancer. A variety of cancer models have established a role for NK cells in tumor immunosurveillance and rejection *in vivo* (Raulet and Guerra, 2009; Wu and Lanier, 2003). NK cells are particularly efficient at preventing blood-borne metastases by eradication of tumor cells present in peripheral circulation in mice (Gorelik et al., 1982). In humans there is often disruption of NK cell function in patients with advanced malignances (Maat et al., 2009) . In recent years, several approaches have been tried to enlist NK cells in the battle against certain cancers in a clinical setting. Specifically attempting to activate a patient's endogenous NK cell subset and adoptive transfer of *in vitro*-activated autologous NK cells have proved successful in certain settings (Laport et al., 2011).

Further understanding of the underlying molecular mechanisms of NK cell responses to cancer will be useful in adapting and enhancing NK cell-based immunotherapy against malignancies. Involvement of various NK cell activating receptors, such as NKG2D (Guerra et al., 2008), NKp46 (Raulet and Guerra, 2009) , and DNAM1 (Chan et al., 2010) , have been implicated in modulating immunity against tumors *in vivo*. However, the anti-tumor NK cell response remains poorly understood. Importantly, the killing of tumors does not seem mediated solely via one receptor, but often seems to rely on a complex repertoire of receptor-ligand interactions (Raulet and Guerra, 2009; Wu and Lanier, 2003).

5 NK cell "Memory"

One major feature used to distinguish adaptive and innate immune responses is the formation of antigen-specific immunological memory. During an immune response, after clonal expansion of antigen-specific T cells, these cells undergo a contraction phase that eliminates more than 90% of the antigen-specific T cells. The cells that still exist after this contraction phase are known as memory cells because they are long-lived, respond more robustly upon secondary infection, and are phenotypically and epigenetically distinct from their naïve counterparts (Min-Oo et al., 2013; Williams and Bevan, 2007). Because NK cells use germ-line-encoded receptors, they were thought to lack antigen-specificity, and because they respond rapidly, NK cells were believed to be incapable of mounting a long-lived memory response.

As mentioned previously, several reports have built a substantial body of evidence demonstrating memory-like features in the NK cell compartment (Cooper and Yokoyama, 2010; Daniels et al., 2001; Dokun et al., 2001; Paust and von Andrian, 2011; Sun et al., 2009, 2010). At least three ways of inducing NK cell memory have been described in mice: (i) antigen-specific NK cell memory induced by MCMV infection; (ii) NK memory cells induced by exposure to cytokines alone; and (iii) liver-restricted NK cell memory with highly antigen-specific recall responses summarized in Figure 7 (Min-Oo et al., 2013). While these three types of memory NK cells are linked by their long-lived phenotype and recall response, each model has shown distinct requirements for organ distribution, effector function, and antigen specificity. However, the underlying molecular mechanisms that control NK cell function and longevity, resulting in distinct effector and memory NK cell subsets during pathogen challenge, are not known. In this thesis, we will focus only on virus-induced memory using a MCMV infection model.

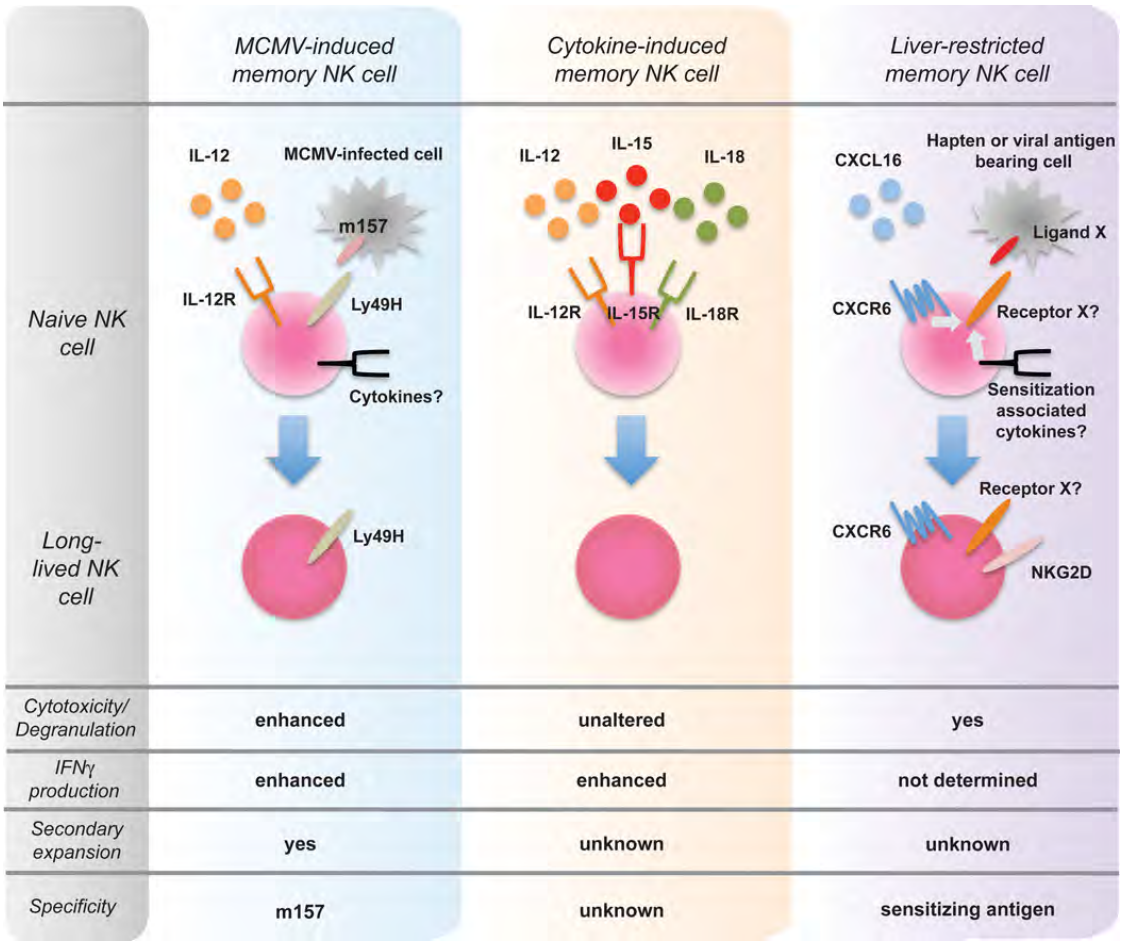
A Virus-induced memory

NK cells have been shown to play a central role in immune-mediated control of MCMV infection (Bukowski et al., 1985; Scalzo et al., 1990; Tay and Welsh, 1997; Welsh et al., 1991). Furthermore, MCMV is the only NK cell infection model where an activating receptor (Ly49H) and its virally encoded cognate antigen (m157) are well characterized (Arase et al., 2002; Brown et al., 2001; Lee et al., 2001; Smith et al., 2002; Tay and Welsh, 1997). MCMV-infected cells present the m157 glycoprotein on their cell surface,

which is recognized by the NK cell activating receptor Ly49H (Arase et al., 2002; Brown et al., 2001; Lee et al., 2001; Smith et al., 2002; Tay and Welsh, 1997) (Figure 8). Upon activation, NK cells release effector cytokines and lytic molecules causing the direct cytolysis of the infected cell. The host antigen-specific Ly49H+ NK cells then undergo clonal-like expansion of effector Ly49H+ NK cells and contraction following viral clearance (Brown et al., 2001; Daniels et al., 2001; Smith et al., 2002). This expansion is critically dependent on the presence of the pro-inflammatory cytokines IL-12 and IL-18, STAT4 and MyD88 signaling, and the transcription factor Zbtb32 (Beaulieu et al., 2014; Madera and Sun, 2015; Sun et al., 2012). The contraction phase forms a long-lived 'memory' pool in lymphoid and non-lymphoid organs that can rapidly respond when the viral antigen is re-encountered (Sun et al., 2009) (Figure 8). Using this mouse model of CMV infection (Sun et al., 2009), we can further investigate the signals that influence NK cell activation, expansion, and memory formation.

Importantly, expansion of NK cells upon infection has also been reported for human patients after human cytomegalovirus (HCMV) infection (Lopez-Verges et al., 2011) (Guma et al., 2006). The selectively expanded subset expressed high levels of the activating receptor CD94-NKG2C. Notably, a long-lived "memory" population of NKG2C-expressing cells persisted in patients and proliferated rapidly upon reactivation (Foley et al., 2012b). Protection by primed NKG2C cells was also transferable (Foley et al., 2012a). During infection of HCMV-seropositive individuals with Hantavirus or chikungunya virus, an NKG2C-positive NK cell subset expanded and persisted until viral

clearance, suggesting a possible role for these cells for the control of various viral infections (Bjorkstrom et al., 2011; Petitdemange et al., 2011).



Reprinted from Trends in Immunology, 34(6), Min-Oo G1, Kamimura Y, Hendricks DW, Nabekura T, Lanier LL. Natural killer cells: walking three paths down memory lane. 251-8. 2013, with permission from Elsevier.

FIGURE 7 | NK CELLS CAN TAKE ONE OF THREE PATHS TOWARDS BECOMING A MEMORY CELL.

Left panel: MCMV-induced NK memory cell is generated after the cognate recognition of the m157 MCMV protein on infected cells by the activating Ly49H receptor. Memory generation requires IL-12 and NK cell signaling through the IL-12R. Additional co-stimulatory signals by other cytokines and/or adhesion molecules might be required. *Center panel*: Cytokine-induced memory NK cells are generated after exposure to IL-12, IL-15, and IL-18. Sensitization with antigen is not required in this model. *Right panel*: Liver-restricted memory NK cell. Sensitization with haptens or specific antigens, in conjunction with CXCL16, is required to generate liver-restricted memory NK cells. The "precursors" might be selected by the cognate recognition of a hapten-modified self-proteins or foreign antigens, and develop into memory NK cells. Receptors responsible for the antigen specificity have not been identified.

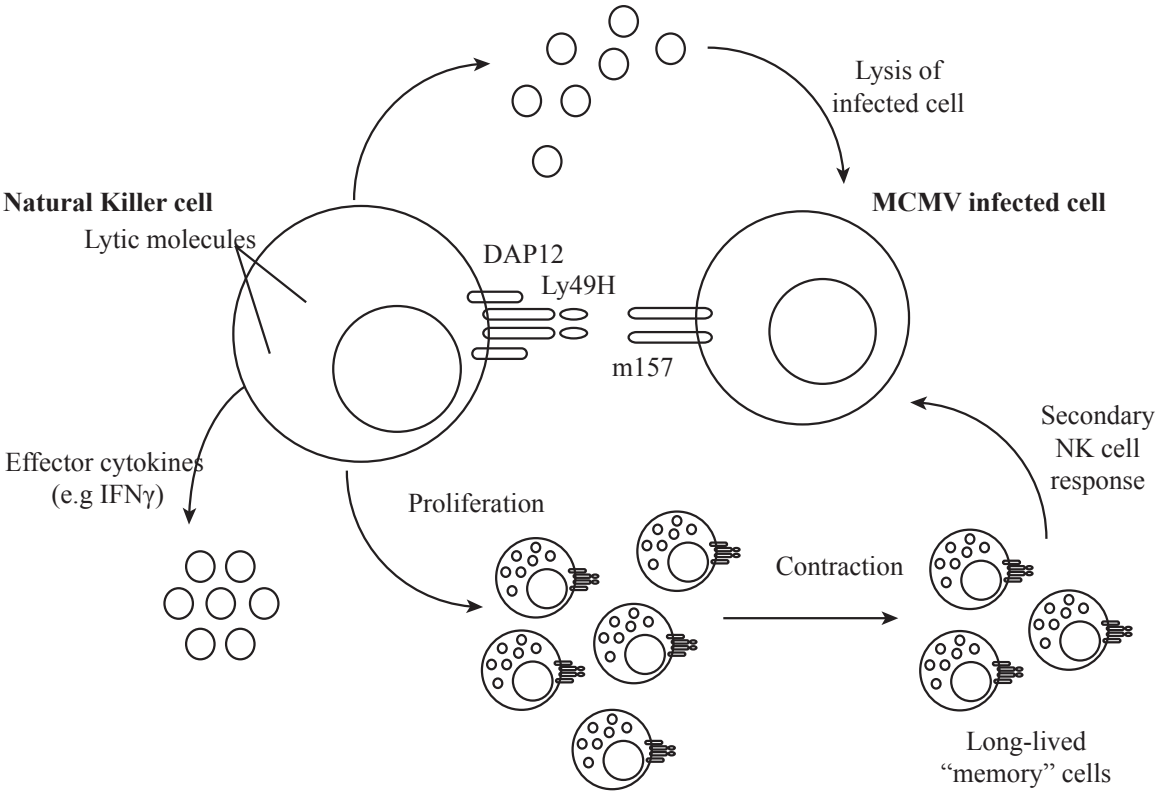


FIGURE 8 | MODEL OF MCMV INDUCED NK CELL MEMORY.

NK cells from B6 mice expressing the activating Ly49H receptor will recognize MCMV-encoded m157 on infected cells, leading to activation and clonal proliferation of antigen-specific NK cells. After the NK cell expansion phase and viral control, effector Ly49H^{+} NK cells undergo a contraction phase resulting in long-lived memory NK cells in lymphoid and non-lymphoid organs. These memory NK cells are able to mount a recall response when virus is re-encountered.

IV SUMMARY

The mammalian immune system has been traditionally subdivided into two compartments known as innate and adaptive. T and B cells, which rearrange their antigen receptor genes using the RAG recombinase, comprise the adaptive arm. Meanwhile, every other white blood cell is merged under the broad umbrella of innate immunity, including NK cells. NK cells are considered innate lymphocytes because of their rapid response and ability to develop without receptor gene rearrangement (i.e. in RAG-deficient mice (Kondo et al., 1997)). However, this classical view of NK cells has been rapidly changing in last decade. Recent evidence suggests that this cell type possesses traits attributable to adaptive immunity (Sun and Lanier, 2011; Vivier et al., 2011), including the ability to generate long-lived "memory" NK cells during viral infection (Daniels et al., 2001; Dokun et al., 2001; Sun et al., 2009, 2010) and phenotypic similarities to CD8+ T cells (Sun and Lanier, 2011). Some of the underlying molecular mechanisms that control NK cell function and longevity, resulting in effector and memory NK cells subsets during pathogen challenge, have only recently come to light.

Although generation of the lymphoid compartment has been studied extensively, unifying models of lymphocyte development have been difficult to construct, and the ontogeny of NK cells is not well understood. Mice that report RAG expression or a history of RAG expression ('fate-mapping' mice) have revealed that a large number of mouse CLPs, which are destined to become B cells or commit to the NK cell lineage, express RAG

(Borghesi et al., 2004; Welner et al., 2009). As expected, all mature B cells exhibit a history of RAG expression; however, a surprisingly large fraction of NK cells (~40%) were also shown to derive from RAG-expressing CLPs (Figure 9). In support of this, independent studies have shown that NK cells can possess nonproductive rearrangements within their Ig and TCR loci (Borghesi et al., 2004; Fronkova et al., 2005; Lanier et al., 1992; Pilbeam et al., 2008). The physiological relevance of RAG expression during NK cell ontogeny has remained unexplored and is the focus of this thesis.

Here we use these RAG fate-mapping and RAG-deficient mice to demonstrate that selective RAG expression during NK cell ontogeny marks functionally distinct subsets of mature NK cells in the periphery. Those NK cells that have a history of RAG expression show an enhanced "fitness" and are capable of greater survival following bursts of robust proliferation. The sum of these results indicate that RAG-mediated DSBs outside of canonical V(D)J recombination in developing lymphocytes may be regulating processes that impact their ultimate survival as mature cells. From these studies a more general paradigm is emerging where activation of the DNA damage response by DSBs generated during timed cell processes (such as transient RAG expression and activity in lymphocyte progenitors) regulate a multitude of cell-type specific programs. This endonuclease-mediated DDR mechanism may be dictating the genomic integrity and fitness of select cells within the total adaptive and innate lymphocyte populations, and ensuring the longevity of specific lymphocyte subsets during periods of rapid proliferation or stress during pathogen invasion.

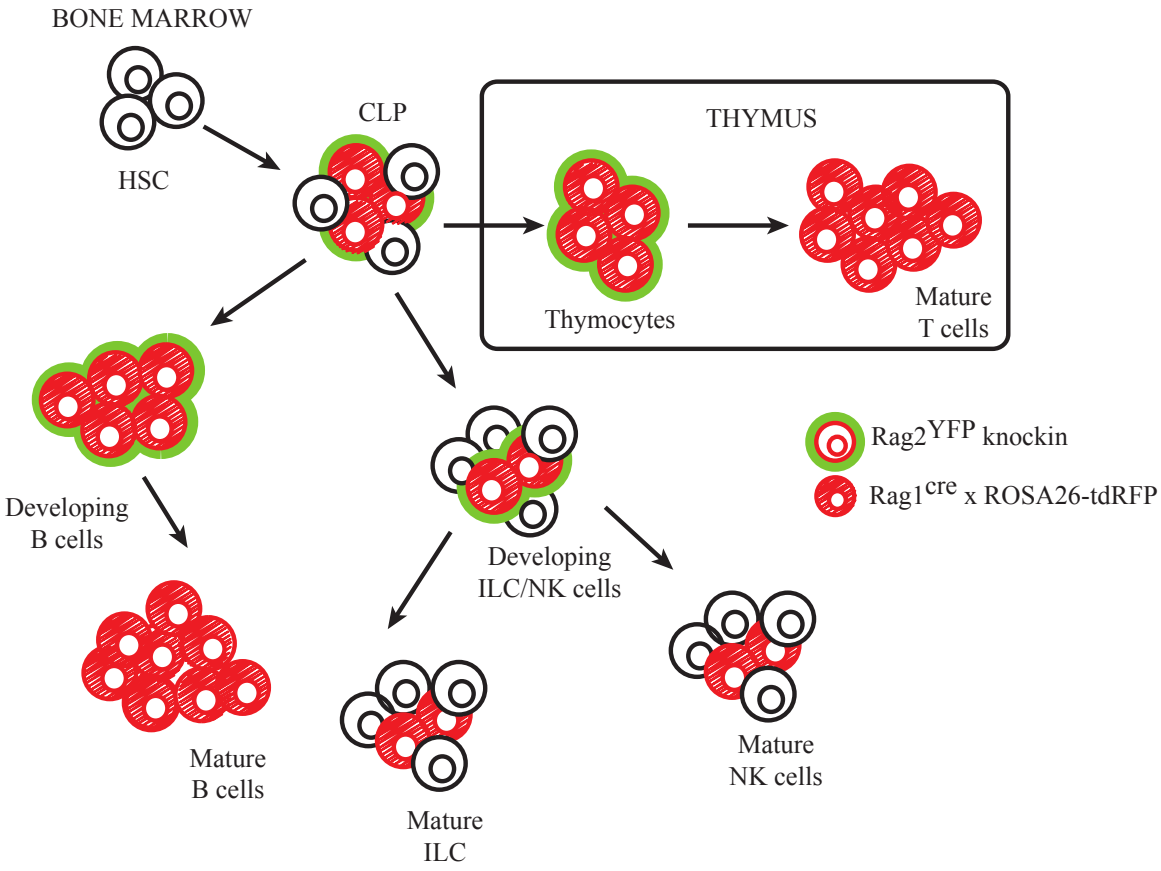


FIGURE 9 | INNATE AND ADAPTIVE LYMPHOCYTES DEMONSTRATING A HISTORY OF RAG EXPRESSION.

Lymphocytes from RAG reporter mice (green outlined cells) and RAG 'fate-mapping' mice (red filled cells) demonstrate that a subset of developing and mature innate lymphoid cells (ILC) and natural killer (NK) cells have a history of RAG gene expression, whereas all mature B and T cells have previously expressed RAG.

CHAPTER 2: MATERIALS AND METHODS

Mice

C57BL/6 (CD45.2+; The Jackson Laboratory), B6.SJL (CD45.1+; Taconic), *Klra8*-/- (Ly49H-deficient)(Fodil-Cornu et al., 2008), *Rag2*$^{-/-}$ (Taconic), *Rag1*$^{-/-}$ (Taconic), SCID (The Jackson Laboratory), nude (The Jackson Laboratory), OT-1 (The Jackson Laboratory), OT-1x*Rag2*$^{-/-}$, *Rag2*$^{-/-}$*xIl2rg*$^{-/-}$ (Taconic), *B2m*$^{-/-}$ (Taconic), *Aid*$^{-/-}$ (Jayanta Chaudhuri), *Xrcc5*-/- and *Xrcc5*+/- (Marilyn Resh), *Atm*$^{-/-}$ (John Petrini), and *Tcrβ*-/- (Sasha Rudensky) mice were maintained at MSKCC, whereas *Rag1*$^{-/-}$ D708A mice were maintained at Yale School of Medicine (David Schatz). *Rag1*^{cre} *x ROSA26*^{tdRFP} and *Rag2*^{GFP} mice were kindly provided by P. Kincade (University of Oklahoma)(McCormack et al., 2003) and P. Fink (University of Washington), respectively..

Mixed bone marrow chimeric mice were generated as described (Sun et al., 2009). Briefly, host C57BL/6 x B6.SJL animals (CD45.1+CD45.2+) were lethally irradiated with 900 grays of radiation and reconstituted with a 1:1 mixture of bone marrow cells from B6.SJL wild-type (WT)(CD45.1+) and knockout donor mice (CD45.2+) mice, co-injected with anti-NK.1 (clone PK136) to deplete any residual donor or host mature NK cells. CD45.1+CD45.2+ host NK cells were excluded from all analyses. Reconstitution was examined in blood 3 weeks post-reconstitution.

For all experiments, mice between 8-10 weeks old were used. All animal work was done in compliance with MSKCC's Internal Animal Care and Use Committee and the guidelines of the Federal Office of Laboratory Animal Welfare. Animals were house in the Research Animal Resources Center (RARC) of MSKCC. Full time veterinarians and technicians were staffed on site and took care of all routine husbandry procedures.

All mice were sacrificed prior to use. Euthanasia was conducted in accordance with the American Veterinary Medical Association Guidelines on Euthanasia. Briefly, mice were sacrificed by asphyxiation with CO_2 delivered into cages <5 pounds per square inch per second. CO_2 euthanasia stations are inspected regularly by the Internal Animal Care and Use Committee personnel (IACUC). Tissues were removed for experiments after confirmation of death.

Virus infections

MCMV (Smith Strain) and MCMV-OVA were obtained from L. Lanier (University of California San Francisco). MCMV was serially passaged through BALB/c hosts 2-3 times, then viral stocks were prepared by using a dounce homogenizer to dissociate the salivary glands of infected mice 3 weeks after infection. Mice were infected with $7.5x10^3$ PFU MCMV by intraperitoneal (i.p.) injection. For the adoptive transfer studies, recipient

Ly49h$^{-/-}$ mice were infected with $7.5x10^2$ PFU MCMV by i.p. injection one day after receiving approximately $5x10^5$ Ly49H$^+$ NK cells.

Flow cytometry and cell sorting

Single cell suspensions were generated from indicated organs and stained with indicated fluorophore-conjugated antibodies (BD Biosciences, eBioscience, BioLegend, Tonbo): NK1.1 (PK136), CD11b (M1/70), CD27 (LG.3A10), CD49b/DX5 (DX5), KLRG1 (2F1), NKp46 (29A1.4), CD69 (H1.2F3), Ly49H (3D10), Granzyme B (16G6), CD107a (1D4B), CD45.1 (A20), CD45.2 (104), CD8α (53-6.7), CD122 (TM-β1), TCRβ (H57-597), CD3ε (145-2C11), IFN-γ (XMG1.2), Ly49D (4E5), Ly49A (A1/Ly49A), Ly49C/I (5E6),T-bet (4B10), Bcl-2 (3F11), Ki67 (B56), GATA-3 (L50-823), RORγT (AFKJS-9), CD19 (6D5), CD4 (L3T4). Gr-1 (RB5-8C5), CD127 (A7R34), CD62L (MEL-14), CD90.2 (30-H12), CD11c (HL3), Flt3 (A2F10.1), Sca-1 (D7), IL-22, IL-23, a4b7. Unless otherwise indicated, NK cells were defined as TCRβ−NK1.1+. For staining OT-1 cells, MHC class I tetramers were generated by conjugating K^b/SIINFEKL monomers (NIH Tetramer Facility) to SA-PE (BD). For staining nuclear proteins and γ-H2AX (Millipore), cells were fixed and permeabilized with the Transcription Factor Staining Kit (eBioscience, Tonbo); with Cytofix/Cytoperm Plus (BD) for staining cytokines; or with formaldehyde and methanol for staining phosphorylated STAT proteins. To maintain RFP expression in *Rag1*^{cre} *x ROSA26*^{tdRFP} mice, cells were fixed for 10 minutes with 1.6% formaldehyde before surface staining.

Pan-caspase staining was carried out using the FAM FLICA TM *in vitro* Poly Caspase Kit (Immunochemistry Technologies). As a positive control, cells were first incubated with 20μg/mL of α-mouse CD95 (clone Jo-2; BD Pharmingen) for 2.5-4 hours at 37˚C (data not shown).

Flow cytometry and cell sorting were performed on the LSR II and Aria II cytometers (BD Biosciences), respectively. For experiments involving qRT-PCR, cell populations were sorted to >95% purity. Data were analyzed with FlowJo software (Tree Star).

NK cell enrichment and adoptive transfer

NK cells were enriched by removing T, B, and red blood cells from total splenocytes suspensions using rat IgG against mouse CD4 (GK1.5), CD8 (53.6.72), CD19 (1D3), and Ter119 (UCSF Core Facility; 10μg of each antibody per spleen) followed by anti-rat IgG-coupled magnetic beads (Qiagen) and magnetic depletion (Sun et al., 2012). Approximately $5x10^5$ enriched NK cells were injected intravenously (i.v.) into adult recipient mice one day before MCMV infection. In some experiments, NK cells or unfractionated splenocytes were labeled with varying concentrations of CFSE before intravenous injection. Labeling of cells with CFSE was performed in accordance with the manufacturer's instructions (Invitrogen).

Ex vivo and in vivo cytotoxicity assays

Enriched NK cells were used as effector cells *ex vivo* in a 6-hour ^{51}Cr release assay against m157-transfected Ba/F3 and Yac1 target cells. Percentage of total and $Ly49H^+$ NK cells in each group was determined by flow cytometry and absolute numbers were normalized before incubation with targets. Large numbers of NK cells from Ly49H-deficient mice and use of untransduced Ba/F3 as target cells were included as negative controls to demonstrate specificity of receptor–ligand interactions (data not shown). *In vivo* cytotoxicity of NK cells was performed by labeling WT and $B2m^{-/-}$ splenocytes with different amounts of CFSE (Invitrogen) and transferring a 1:1 WT: $B2m^{-/-}$ mixture into $Rag2^{-/-}$ or WT recipient mice. Percent lysis of transferred cells was determined at various time points indicated.

Ex vivo NK cell stimulation

Enriched NK cells or whole splenocytes were incubated for 5 h at 37°C in the presence of GolgiPlug (BD) and RPMI containing 5% FBS with recombinant mouse IL-12 (20 ng/ml; R&D Systems) or IL-18 (10 ng/ml; R&D Systems) or with plate-bound antibodies (10 μg/ml; eBioscience) against the activating NK cell receptors Ly49H (3D10), Ly49D (4E5), NKp46 (29A1.4) or NK1.1 (PK136). Cells were stained for intracellular cytokines. Uncoated or PBS-coated wells served as negative controls, and addition of 50 ng/ml PMA and 1 μg/ml ionomycin during incubation served as a positive control.

For *ex vivo* NK cell expansion experiments, 2.5×10^5 sorted NK cells (>98% purity) were stimulated in RPMI supplemented with 10% FBS, penicillin-streptomycin, l-glutamine and β-mercaptoethanol, and containing recombinant mouse IL-2 (20 ng/ml; R&D Systems) and IL-15 (40 ng/ml; R&D Systems). Fresh medium and cytokines were added on alternating days of culture.

Ex vivo T cell stimulation

Equal numbers of OT-1 (CD45.1) and OT-1 x *Rag2*$^{-/-}$ (CD45.2) T cells were co-cultured in complete RPMI + 10% DBS with 1:1000 2-mercaptoethanool (Sigma) containing 1 μM SIINFEKL peptide or anti-CD3/28 beads (Life Technologies).

BrdU treatment and staining

Mice were given BrdU (5-Bromo-2′-Deoxyuridine) (Sigma) (0.8 mg/mL) in the drinking water for two weeks ("pulse"), and then given normal drinking water ("chase"). After various periods of time on normal drinking water, isolated lymphocytes from labeled mice were first stained for expression of surface markers, and after fixation, the cells were then stained with an anti-BrdU mAb (BD Biosciences).

Quantitative RT-PCR

Sorted NK cells were lysed in Tri-Reagent (Ambion). RNA purification and cDNA synthesis were carried out with the Qiagen RNeasy kit (with on-column DNase I treatment), and MulV reverse transcriptase and Oligo $(dT)_{16}$ primers (Applied Biosystems). iQ Sybr Green SuperMix (BioRad) was used for qRT-PCR. Data were normalized to *Actb* and expressed as relative target abundance via the ΔΔCT method, where Ct (threshold cycle) is the cycle number at which the amplification curve intersects the threshold value Table 1 lists relevant primer sequences.

Table 1:

	Forward	**Reverse**
DNA PK	ATAGAAGTAGACTCGCCAAG	ACCCAGAGATGATAAGTCAC
Ku 80	ACCAAGTGACTGCTCAGGAC	AGGCTGGAGATGCTGATGTG
ATM	CGAGCACAAGATTCAGCTGC	ACTGGTAACCTGGGCCATTG
CHEK2	ATCATCAGTCCCACAGCAGC	TGCAACTGAGAAGGAGTGCC

Isolation of ILC subsets and ex vivo stimulation

Spleens, MLNs, and Peyer's patches were mechanically crushed into single cell suspensions. Lungs, intestines, and fat were digested in collagenase type 4 (Worthington), collagenase D (Roche), and collagenase type 2 (Worthington), respectively. To assess production of cytokines, ILC2 and ILC3 cells were stimulated for

3 h at 37°C in complete RPMI + 10% FBS with 1:1,000 Brefeldin A (BD), 1:1,000 2-mercaptoethanol (Sigma-Aldrich), and 40 ng/ml IL-23 (for ILC3 stimulation) or 0.1 μg/ml PMA + 1 μg/ml ionomycin (for ILC2 stimulation), followed by intracellular staining. Unstimulated controls (media only) were used to determine gating strategy for flow cytometric plots.

C. rodentium infections and titers

In *C. rodentium* studies, mice were inoculated by oral gavage with 10^8 CFU (in 200 μl) of an overnight LB culture of *C. rodentium* (strain DBS100). Infected mice were assessed for body weight, signs of morbidity, and bacterial titers. To determine *C. rodentium* titers, fecal or cecal contents were mechanically homogenized in PBS and 10-fold serial dilutions cultured overnight on MacConkey's agar, as previously described (Sonnenberg et al., 2011).

Chromatin immunoprecipitation (ChIP)

~5 × 10^6 enriched splenic NK cells were purified by sorting on TCRβ−NK1.1+ cells. Proteins were cross-linked to DNA for 8 min at 25 °C by addition of 1% formaldehyde to the medium. ChIP was carried out as previously described (Zheng et al., 2007) using 10 μg of rabbit polyclonal anti-H3K4me3 (Abcam,) or 10 μg of rabbit serum IgG antibody as a control (data not shown; Sigma, I5006). Relative abundance of regulatory sequences in the *Chek2* promoter or, as controls, in the *Gapdh* promoter or a 'gene desert' ~50 kb

upstream of the Foxp3 gene was measured by qPCR in the antibody precipitated DNA using iQ Sybr Green SuperMix (BioRad). After determining the Ct value, percent input was calculated as $100 \times 2^{(\text{Ct adjusted input} - \text{Ct target})}$, where the Ct^{input} was adjusted from 5% to 100% by subtracting log2 20 Ct values. Primer sequences are listed in Table 2:

Table 2:

Gene Target	**Forward Primer**	**Reverse Primer**
pChek2	AGCCTTGAGACTAATCGCGG	ACGGATACAAACTCCACCCTC
pChek2	TGAGGGTGGAGTTTGTATCCG	TCCCACTTATGACTCACCGC
pChek2	CCGTTCTCATGCTTCTTGTGC	AGAATTGACTATGCACAGCGC
pGapdh	TGAGTCCTATCCTGGGAACCATCA	TTTGAAATGTGCACGCACCAAGCG
Gene Desert*	TAGCCAGAAGCTGGAAAGAAGCCA	TGATACCCTCCAGGTCCAACCATT

*(50kB upstream *pFoxp3*)

Immunofluorescence to detect irradiation induced foci

Sorted WT and Rag2-deficient NK cells were seeded on Poly-L-Lysine treated coverslips using cytospin. Cells were washed with PBS and fixed with 3% PFA+2% sucrose at room temperature (RT). Fixative was removed and cells were washed at PBS and kept at 4˚C. Cells were then permeabilized with PBS+0.5% TX-100 (1% solution in PBS) for 10 min at RT and blocked for 30 min RT with PBS+ 5% FBS + 1% BSA. Cells were incubated with primary antibody (anti-YH2AX (Millipore) at 1:5000 dilution with 1% BSA) for 2h RT. Cells were washed 5-6 times with PBS before the addition of secondary

antibody (anti-mouse Alexa488 (Invitrogen) at 1:1000) for 1h RT. Cells were washed with PBS and DAPI (5mg ml – 1 stock diluted 1:5000 in PBS; Invitrogen) was added for 5 min. Cells were washed and mounted for 40 min with ProLong Gold Antifade mounting media (Molecular Probes). Samples were stored at 4˚C overnight and read using an upright Zeiss Axio Z1 microscope. Images were acquired with an AxioCam MRn camera using AxioVision 4.8.

B16 tumor challenge

$1x10^6$ WT or KO enriched NK cells were adoptively transferred into *Rag2*-/-*xIL2γc*-/- hosts at d0. At d1 $2x10^5$ B16 tumor cells (Ming Li) were transferred intravenously (i.v.). Hosts were monitored for several key aspects of tumorigenesis, such as: weight loss, survival, and tumor burden. As a control, WT mice were treated with NK depletion antibody (a-PK136). Lung and liver colonization was examined at d21 post tumor challenge.

Statistical analysis

For graphs, data are shown as mean ± s.e.m. and, unless otherwise indicated, statistical differences were evaluated using a two-tailed unpaired Student's t-test, assuming equal sample variance. $P < 0.05$ was considered significant. Statistical differences in survival were determined by Gehan-Breslow-Wilcoxon Test analysis. Graphs were produced and statistical analyses were performed using GraphPad Prism. Sample size was not

specifically predetermined, but the number of mice used was consistent with prior experience with similar experiments.

CHAPTER 3: RESULTS I

Heterogeneous NK cell subsets distinguished by RAG expression

Introduction

The Recombination Activating Gene proteins RAG1 and RAG2 (collectively, RAG) mediate V(D)J gene rearrangement at the antigen receptor loci during lymphocyte development, giving rise to lymphocytes with unique specificity and providing the molecular mechanism behind Burnet's theory of clonal selection. In contrast to T and B cells, NK cells classically represent a third lineage of lymphocytes that possess germ-line-encoded antigen receptors and do not require receptor gene rearrangement for their development (Kondo et al., 1997). Given that NK cells do not require V(D)J recombination or express surface immunoglobulin (Ig) or T cell receptor (TCR) proteins, they are not thought to require RAG proteins for their development, function, or survival. Indeed, NK cells are present in normal numbers in RAG-deficient mice, whereas T and B cells are completely absent (Mombaerts et al., 1992; Shinkai et al., 1992). Currently, there is no evidence that RAG plays a physiological role in any cell type other than B and T lymphocytes or in any process other than V(D)J recombination.

While generation of the lymphoid compartment has been studied for many years, unifying models of lymphocyte development have been difficult to construct, and the ontogeny of NK cells is not well understood. Mice that report RAG expression or a history of RAG expression ('fate-mapping' mice) have revealed that a large number of mouse CLP, destined to become B cells or commit to the NK cell lineage, express RAG at some point during development (Borghesi et al., 2004; Welner et al., 2009). As expected, all mature B cells exhibit a history of RAG expression (RFP+); however, a surprisingly large fraction of NK cells (~40%) also derive from RAG-expressing CLPs (Figure 9). In support of this, independent studies have shown that NK cells can possess nonproductive rearrangements within their Ig and TCR loci (Borghesi et al., 2004; Fronkova et al., 2005; Lanier et al., 1992; Pilbeam et al., 2008). The physiological relevance and consequence of RAG expression during NK cell ontogeny has remained unexplored.

Here we use RAG fate-mapping and RAG-deficient mice to demonstrate that selective RAG expression during NK cell ontogeny gives rise to functionally heterogeneous populations of effector cells within the peripheral NK cell pool. Specifically we saw 20-30% of peripheral NK cells have at one point expressed RAG (i.e. are marked by RFP expression) (Borghesi et al., 2004; Ichii et al., 2010; Karo et al., 2014; Lanier et al., 1992; Pilbeam et al., 2008) (Figure 9). Analysis of NK cell development in RAG reporter mice (Igarashi et al., 2001; Kuwata et al., 1999) revealed highest levels of RAG expression in CLP from bone marrow, with GFP expression decreasing as developing NK cells undergo maturation and migration from bone marrow. NK cells that never expressed

RAG during development (RFP^-) were more terminally differentiated (as defined by greater KLRG1 expression), and demonstrated a higher degree of cytotoxicity (Karo et al., 2014). Further, we investigated whether RAG influenced *in vivo* NK cell responses by incorporating a well-established viral model of antigen-specific NK cell expansion (Sun et al., 2009) and saw NK cells with a history of RAG expansion survive better post viral infection. Together, these data demonstrates that RAG expression in CLPs and NK cell precursors distinguishes subsets of mNK cells that are functionally distinct.

Results

Selective RAG expression during ontogeny distinguishes functional heterogeneity within the NK cell population

Using RAG fate-mapping mice ($Rag1^{Cre}$ x Rosa26-floxed STOP-tdRFP) (Welner et al., 2009), we observed that 40-50% of bone marrow NK cells and 20-30% of peripheral NK cells have at one point expressed RAG1 (i.e. are marked by RFP expression) (Figure 10), consistent with previous findings (Borghesi et al., 2004; Ichii et al., 2010; Lanier et al., 1992; Pilbeam et al., 2008). Analysis of the NK lineage in $RAG2^{GFP}$ knockin reporter mice (Igarashi et al., 2001; Kuwata et al., 1999) revealed highest levels of RAG expression in common lymphoid progenitors (CLP) from bone marrow, with expression decreasing as NK cells undergo maturation (Figure 11A & B). Interestingly, NK cells that lack evidence of prior RAG expression during development (RFP^-) were more activated and terminally differentiated (i.e. higher KLRG1 expression) in all organs

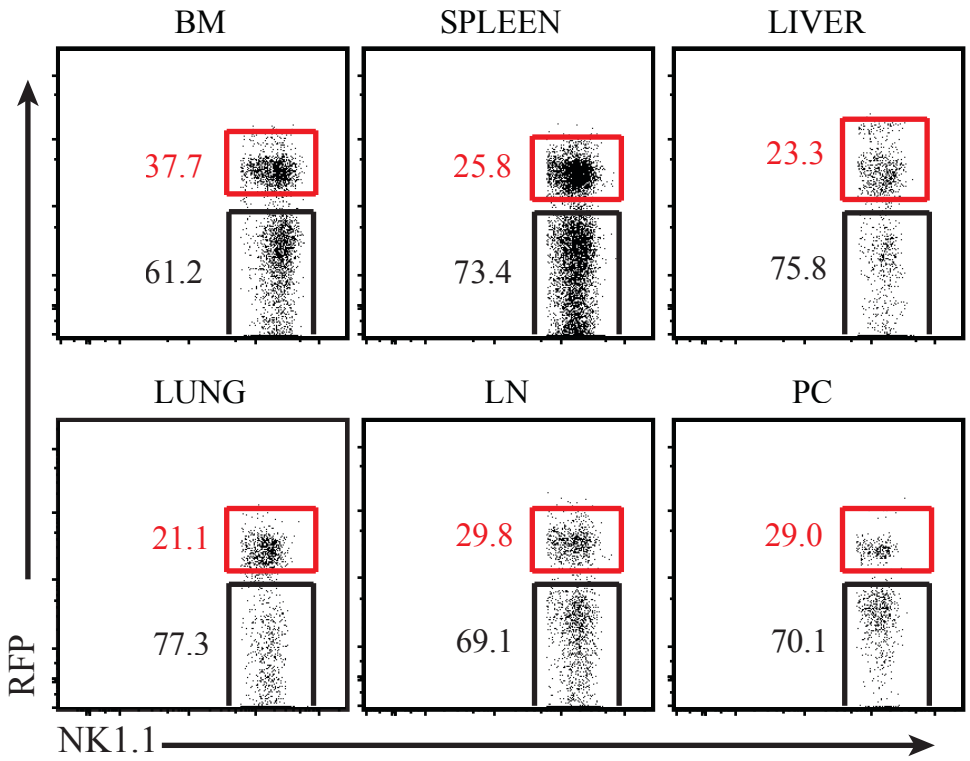


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FIGURE 10 | PERIPHERAL NK CELLS HAVE A HISTORY OF RAG EXPRESSION.

$NK1.1^{+}$ $TCR\beta^{-}$ NK cells from various organs of $Rag1^{Cre}$ x $ROSA^{TdRFP}$ mice were analyzed for RFP expression (red). BM: bone marrow; LN: lymph node; PC: peritoneal cavity.

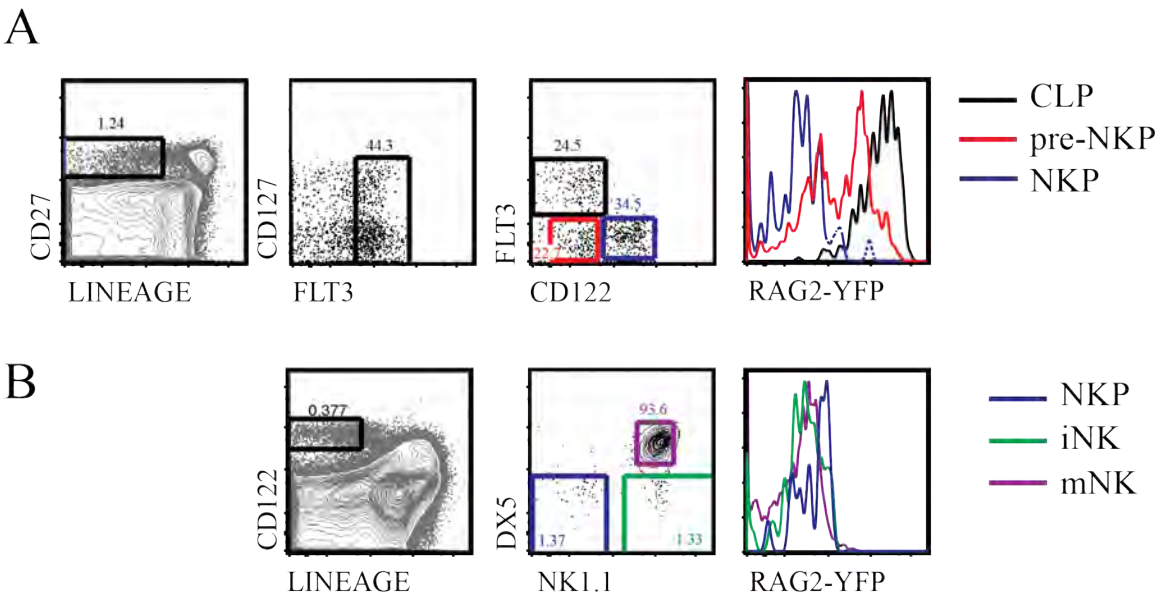

FIGURE 11 | RAG EXPRESSION IS SEEN AT CLP DEVELOPMENTAL STAGE.

(A-B) Flow cytometric analysis of GFP expression was performed on bone marrow of *Rag2*^{GFP} mice using FLT3 and CD122 to identify CLP (FLT3^{+} CD122^{-}), pre-NKP (FLT^{-} CD122^{-}), and rNKP (FLT3^{-} CD122^{-}) within the Lin^{-} CD27^{+} CD127^{+} cell population (A); and NK1.1 and DX5 were used to identify NKP (NK1.1^{-}DX5^{-}), iNK (NK1.1^{+} DX5^{-}), and mNK (NK1.1^{+} DX5^{+}) cells within the Lin^{-} CD122^{+} population (B). Lin^{-} (or Lineage-negative) is defined as CD19^{-} CD3^{-} TCRβ^{-} CD4^{-} CD8^{-} Ter119^{-}. Gating strategy shown.

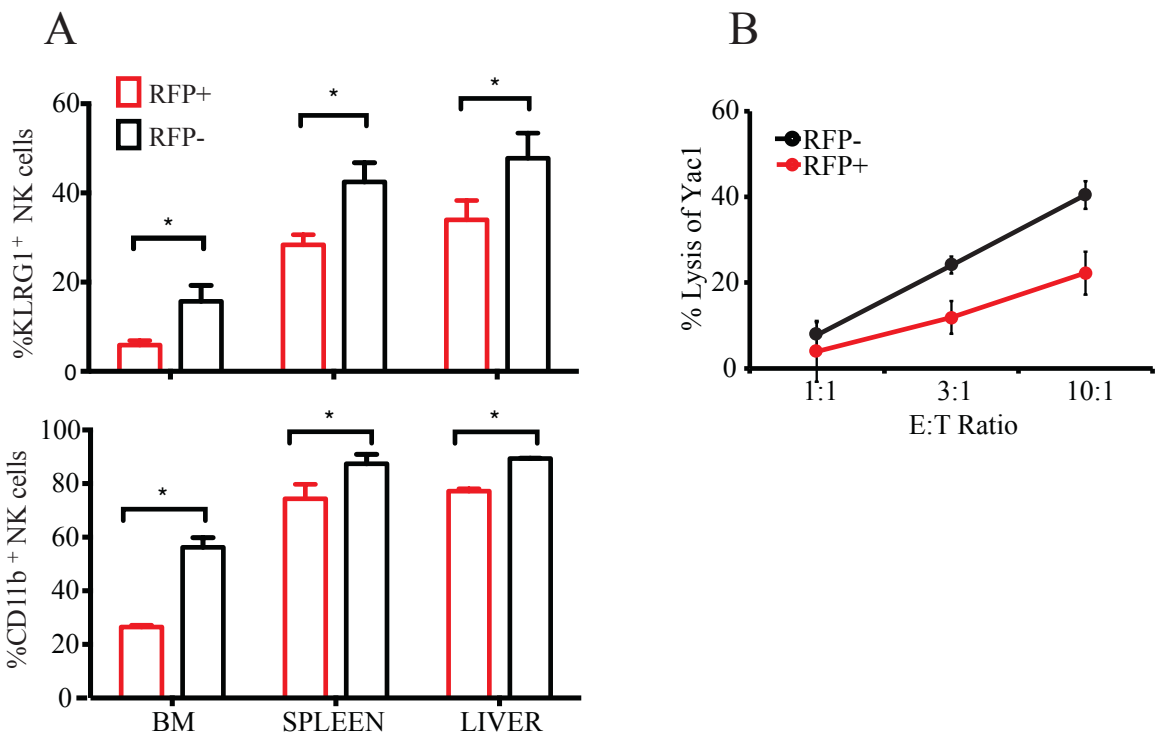

FIGURE 12 | HISTORY OF RAG EXPRESSION DELINEATES HETEROGENEITY WITHIN THE NK CELL POPULATION.

(A) NK1.1^{+} TCRβ^{-} NK cells from various organs of *Rag1*^{Cre} *x ROSA*^{TdRFP} mice were analyzed for activation and maturation markers (KLRG1 and CD11b). Error bars for all graphs show s.e.m. and all data are representative of n=3-4 mice in three independent experiments. *$p < 0.01$ **(B)** Percent lysis of ^{51}Cr-labeled Yac1 target cells by sorted RFP^{+} (solid line) and RFP^{-} (dashed line) NK cells *ex vivo*. Data representative of three independent experiments performed with n=3-5 mice.

(Figure 12A), and demonstrated a higher degree of cytotoxicity (Figure 12B), as compared to NK cells that had expressed RAG (RFP^+). Given that peripheral NK cells do not express RAG (determined by $RAG2^{GFP}$ reporter mice, Figure 13), our data demonstrate that selective RAG expression during NK cell ontogeny correlates with functionally distinct progeny cells and heterogeneity in the peripheral NK cell pool.

RAG-deficiency results in NK cells with a cell-intrinsic hyper-responsiveness

We next investigated whether NK cells lacking *Rag* were phenotypically and functionally similar to RFP^- NK cells in the fate-mapping mice. Mice deficient in *Rag2* lack peripheral T and B cells (Shinkai et al., 1992), but contain normal NK cell numbers compared to wild-type (WT) mice (Figure 14A). Nevertheless, $Rag2^{-/-}$ NK cells exhibited a more mature ($KLRG1^{hi}$, $CD27^{lo}$, $CD11b^{hi}$) and activated ($CD69^{hi}$, $CD62L^{lo}$) phenotype at steady-state when compared to WT NK cells (Figure 14B & 5C), consistent with a previous report (Andrews and Smyth, 2010). Furthermore, similar to RFP^- NK cells in the fate-mapping mice, $Rag2^{-/-}$ NK cells were more cytotoxic on a per cell basis *in vitro* and *in vivo* compared to WT NK cells (Figure 15). A similar hyper-responsiveness was observed in $Rag2^{-/-}$ NK cells in mixed bone marrow chimeric mice (Figure 16A), where lethally irradiated WT mice were injected with equal numbers of WT (CD45.1) and $Rag2^{-/-}$ (CD45.2) bone marrow cells and allowed to reconstitute the hematopoietic compartment (Figure 16B). We noted that the phenotype of $Rag2^{-/-}$ NK cells in the mixed chimera setting was not as pronounced as in NK cells isolated directly from $Rag2^{-/-}$ mice,

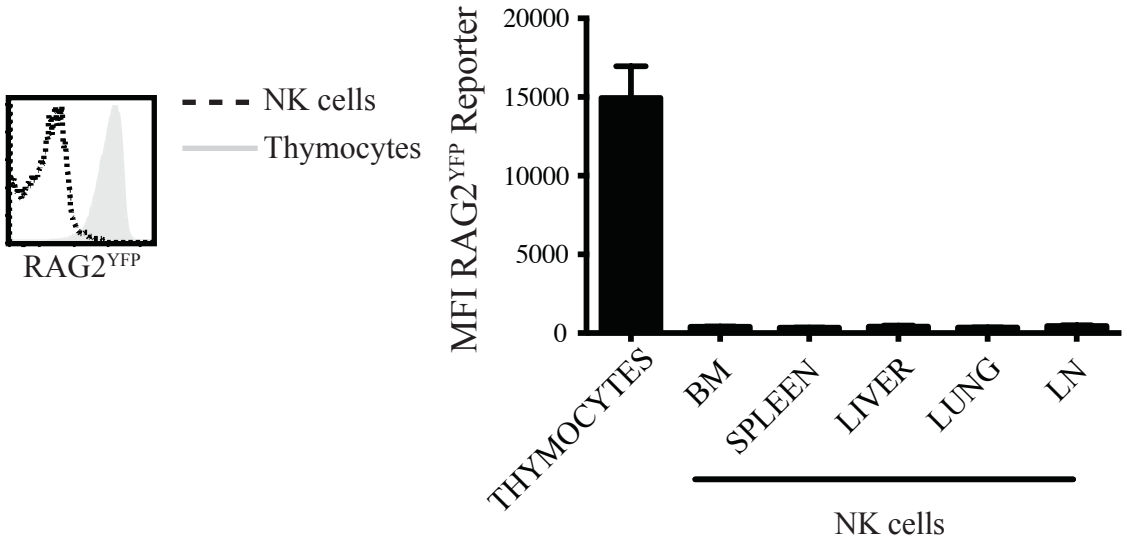

FIGURE 13 | RAG IS NOT EXPRESSED IN MATURE PERIPHERAL NK CELLS.

NK1.1^{+} TCRβ^{-} NK cells from various organs of *RAG2^{GFP}* reporter mice were analyzed for GFP expression at steady state and compared to TCRβ^{+} thymocytes. Quantification and a representative histogram are shown. Results shown are representative of three independent experiments (n=3-4 mice).

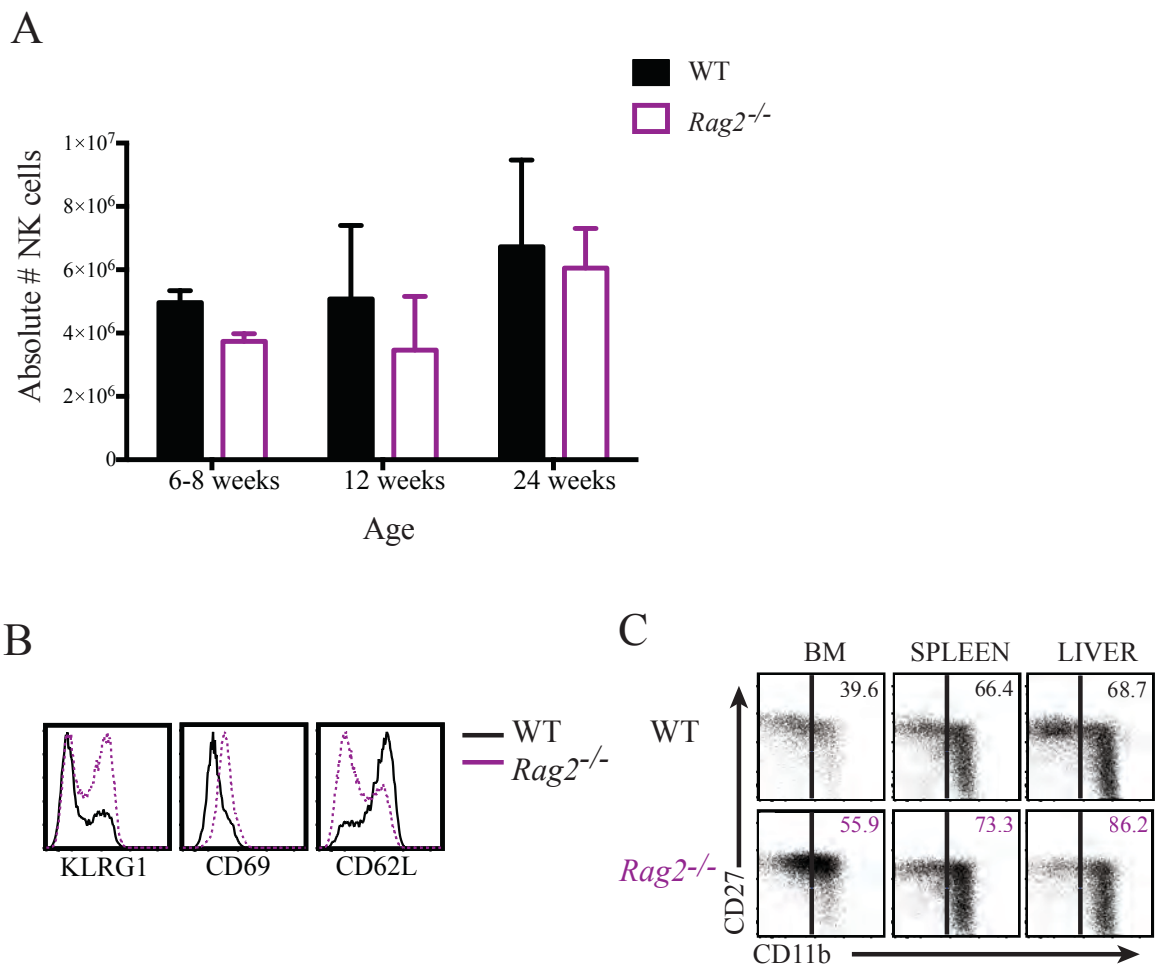

FIGURE 14 | LACK OF RAG PRODUCES A HYPER-RESPONSIVENESS IN NK CELLS.

(A) Absolute numbers of splenic NK cells from WT and *Rag2*$^{-/-}$ mice at various ages. Error bars show s.e.m. and data are representative of at least three independent experiments performed with n=3-5 mice. **(B-C)** Splenic NK cells from wild type (WT) and Rag2-deficient (*Rag2*$^{-/-}$) mice were analyzed for the activation and maturation markers KLRG1, CD69, CD62L, CD27, and CD11b.

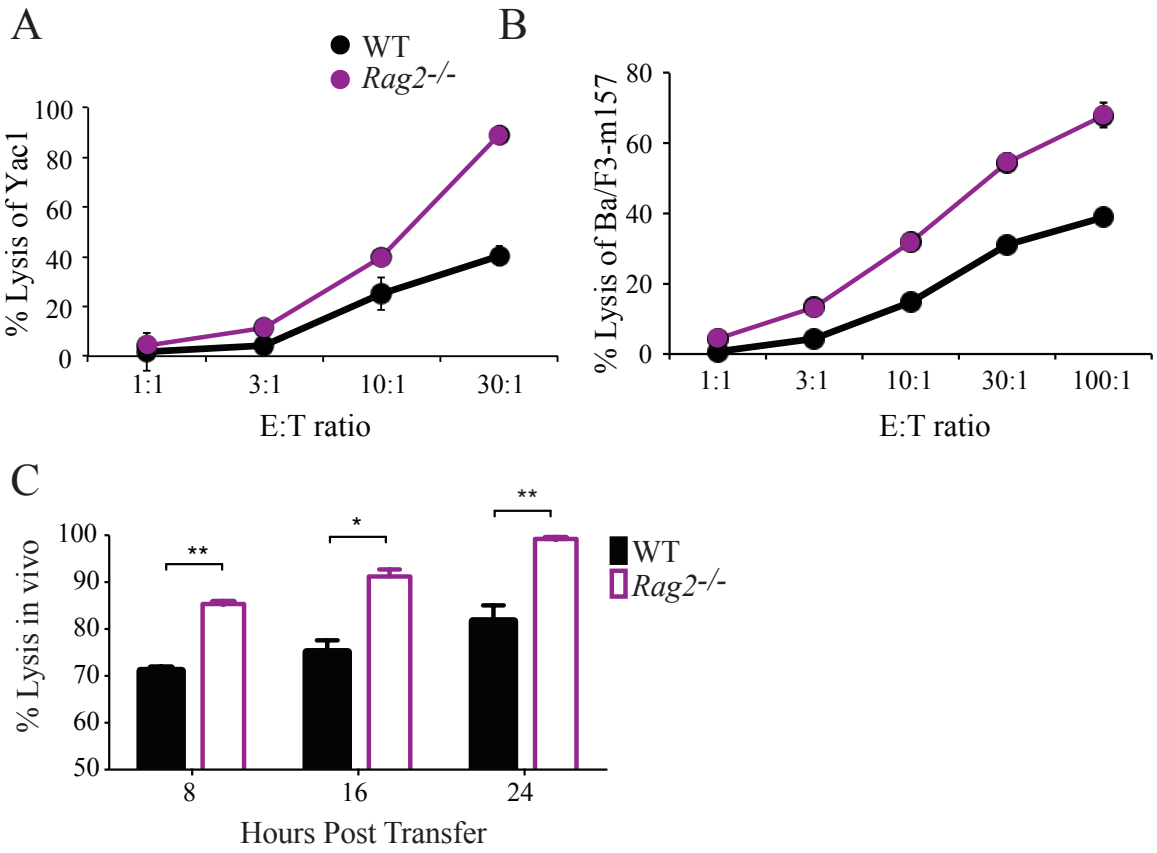

FIGURE 15 | LACK OF RAG PRODUCES CYTOTOXIC NK CELLS.

(A-B) Percent lysis of ^{51}Cr-labeled Yac1 (A) and Ba/F3-m157 (B) target cells by WT and *Rag2*$^{-/-}$ NK cells *ex vivo*. Data are representative of three independent experiments performed with n=3-5 mice. (**C**) Percentage of CFSE-labeled β2m-deficient target cells lysed by NK cells *in vivo* in WT and *Rag2*$^{-/-}$ mice at indicated time points after transfer. Error bars show s.e.m. and the data depicts 3-5 mice per group, repeated in three independent experiments. *p < 0.05; **p < 0.005. **(D-E)** Percent lysis of ^{51}Cr-labeled Yac1 target cells by WT and *Rag2*$^{-/-}$ compared to B- (D) and T- (E) cell deficient NK cells *ex vivo*.

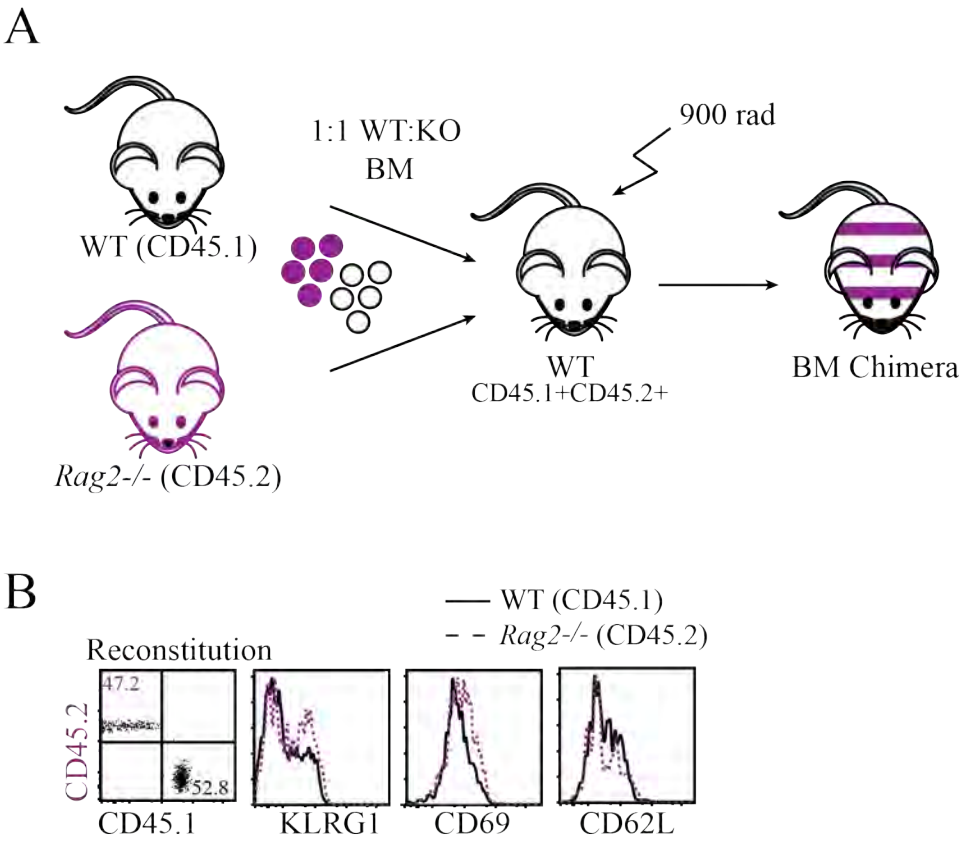

FIGURE 16 | *RAG*-DEFICIENT HYPER-RESPONSIVENESS IN NK CELLS IS CELL-INTRINSIC.

(A-B) Lethally-irradiated hosts (CD45.1xCD45.2) were injected with equal numbers of WT (CD45.1) and *Rag2*$^{-/-}$ (CD45.2) bone marrow cells (Schematic, A). 8-12 weeks following hematopoietic reconstitution, NK cells were analyzed for the activation markers shown (B). Data are representative of at least three independent experiments performed with n=3-5 mice.

suggesting that both intrinsic and extrinsic factors might contribute to NK cell hyper-responsiveness in unirradiated *Rag2*$^{-/-}$ mice. Similar observations were made in NK cells from *Rag1*$^{-/-}$ mice (Figure 17), however TCRβ-deficient, and AID-deficient mice behaved like WT NK cells (Figure 18).

RAG-deficient NK cells fail to expand and persist following MCMV infection

To investigate whether RAG influences other NK cell functions, we used a well-established viral model of antigen-specific NK cell expansion (Sun et al., 2009). In this model, NK cells expressing the mouse cytomegalovirus (MCMV)-specific activating receptor Ly49H undergo robust antigen-driven proliferation (100-1000 fold expansion) following MCMV infection, and following viral clearance, a population of long-lived memory NK cells persists in both lymphoid and non-lymphoid organs (Sun et al., 2009). When equal numbers of splenic WT (CD45.1) and *Rag2*$^{-/-}$ (CD45.2) Ly49H-expressing NK cells were adoptively co-transferred into Ly49H-deficient mice followed by infection with MCMV (Figure 19A), the WT NK cells expanded more robustly than *Rag2*$^{-/-}$ NK cells in the first 7 days post-infection (PI), and continued to persist at a greater percentage for over a month (Figure 19B-D). The defect in *Rag2*$^{-/-}$ NK cell expansion during MCMV infection was observed in all organs (Figure 19E), demonstrating that diminished *Rag2*$^{-/-}$ NK cell numbers in the peripheral blood was not due to aberrant trafficking or retention in non-lymphoid tissues. Donor NK cells from *Rag1*$^{-/-}$ mice were similarly outcompeted by WT NK cells following adoptive transfer and MCMV infection (Figure 20A-B) in both lymphoid and non-lymphoid organs (Figure 20C). WT NK cells outcompeted RAG-

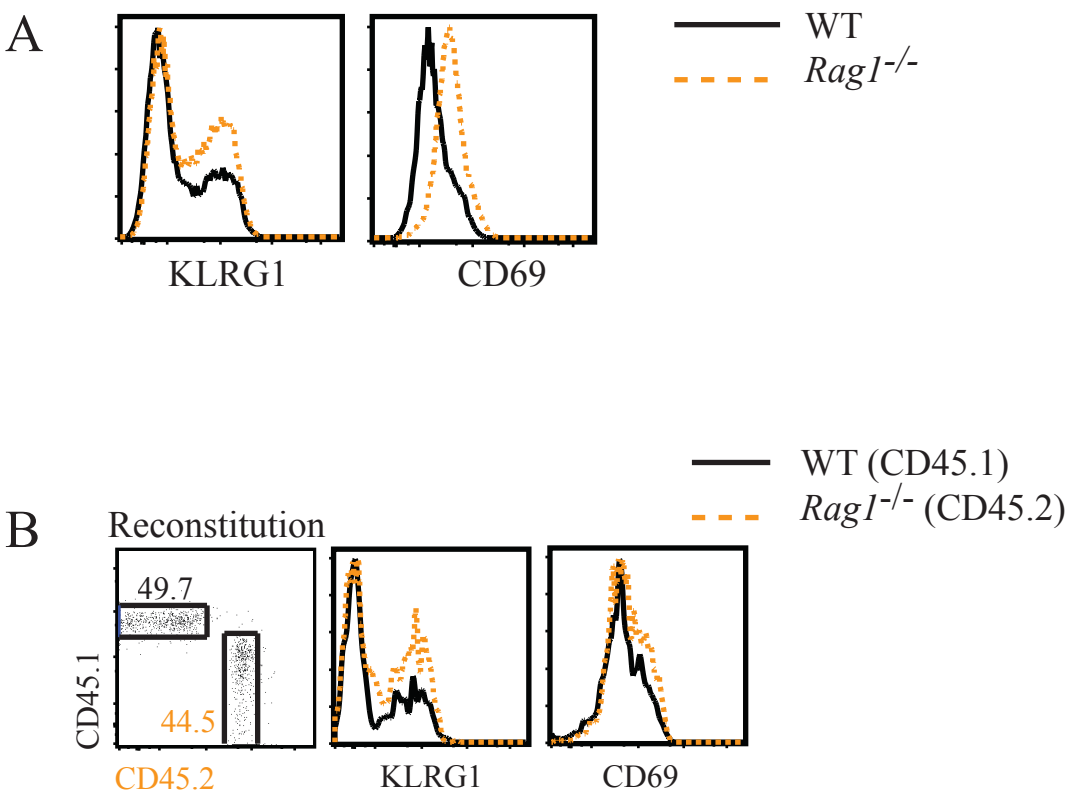

FIGURE 17 | LACK OF *RAG1* PRODUCES A CELL-INTRINSIC HYPER-RESPONSIVENESS IN NK CELLS.

(A) Splenic NK cells from WT and *Rag1*$^{-/-}$ mice were analyzed for the activation markers shown. **(B)** Lethally-irradiated mice were injected with equal numbers of WT (CD45.1) and *Rag1*$^{-/-}$ (CD45.2) bone marrow cells. 8-12 weeks following hematopoietic reconstitution, NK cells were analyzed for the activation markers shown. Data are representative of at least three independent experiments performed with n=3-5 mice.

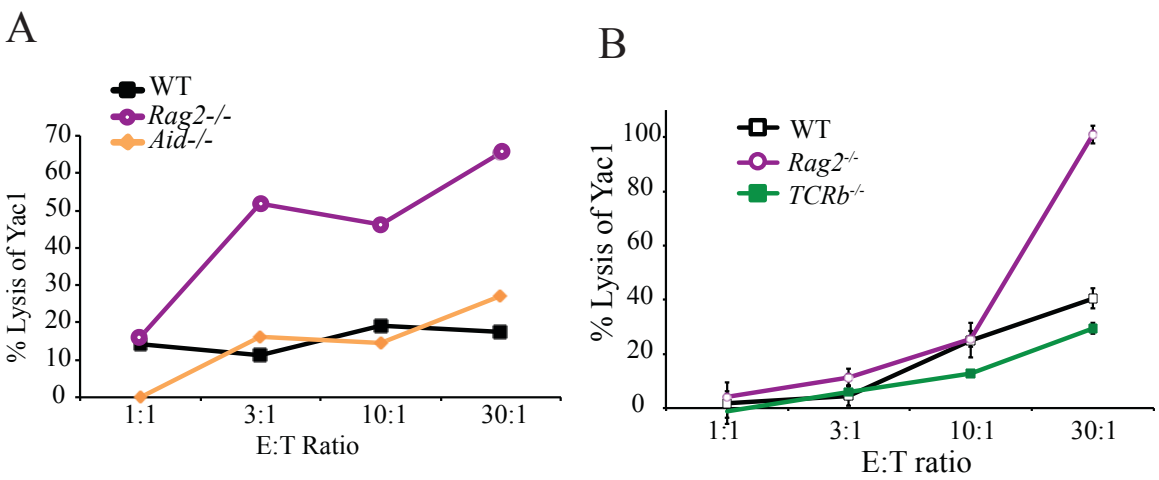

FIGURE 18 | HYPER-RESPONSIVENESS IS NOT DUE TO ABSENCE OF T OR B CELLS.

(A-B) Percent lysis of ^{51}Cr-labeled target cells by WT (black), Rag2$^{-/-}$ (purple) and B cell (*Aid*$^{-/-}$) (A) or T cell (*Tcrβ*$^{-/-}$) (B) – deficient NK cells *ex vivo*. Data are representative of three independent experiments performed with n=3-5 mice.

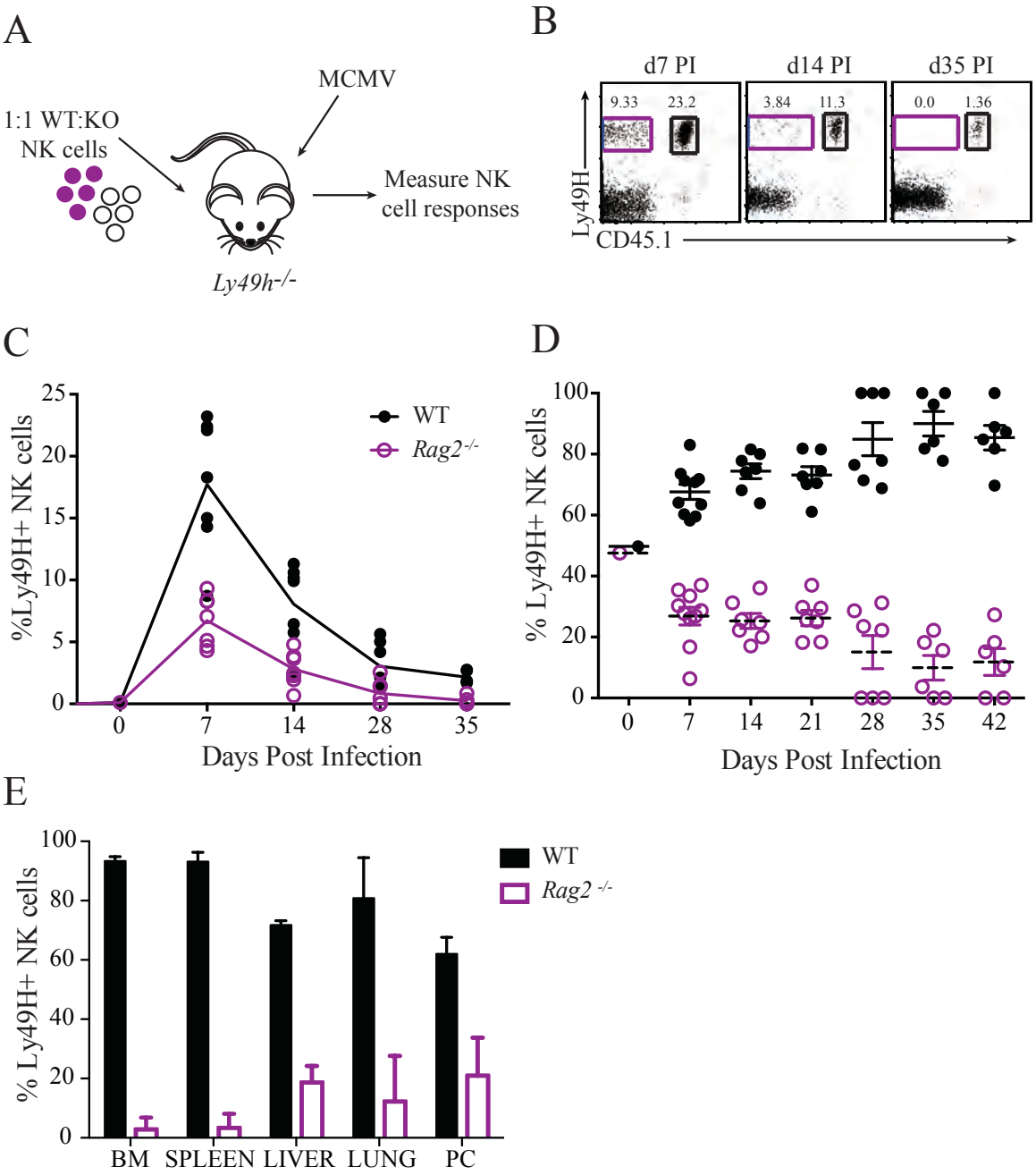

FIGURE 19 | *RAG*-DEFICIENT NK CELLS FAIL TO EXPAND FOLLOWING MCMV INFECTION.

(A) Schematic of experiment. Equal numbers of WT (CD45.1) and *Rag2*$^{-/-}$ (CD45.2) Ly49H^{+} NK cells were co-transferred into Ly49H-deficient (*Ly49h*$^{-/-}$) mice prior to infection with MCMV. **(B-D)** The absolute and relative percentages of adoptively transferred WT

versus *Rag2*$^{-/-}$ Ly49H^{+} NK cells in peripheral blood at various time points following MCMV infection are shown. Data are representative of five independent experiments (n=5-10 mice per time point), with each data point representing an individual mouse. **(E)** Graph shows percentage of transferred WT and *Rag2*$^{-/-}$ Ly49H^{+} NK cells in indicated organs at day 10 PI. Error bars show s.e.m. and the graph is representative of three independent experiments with 4-5 mice per group.

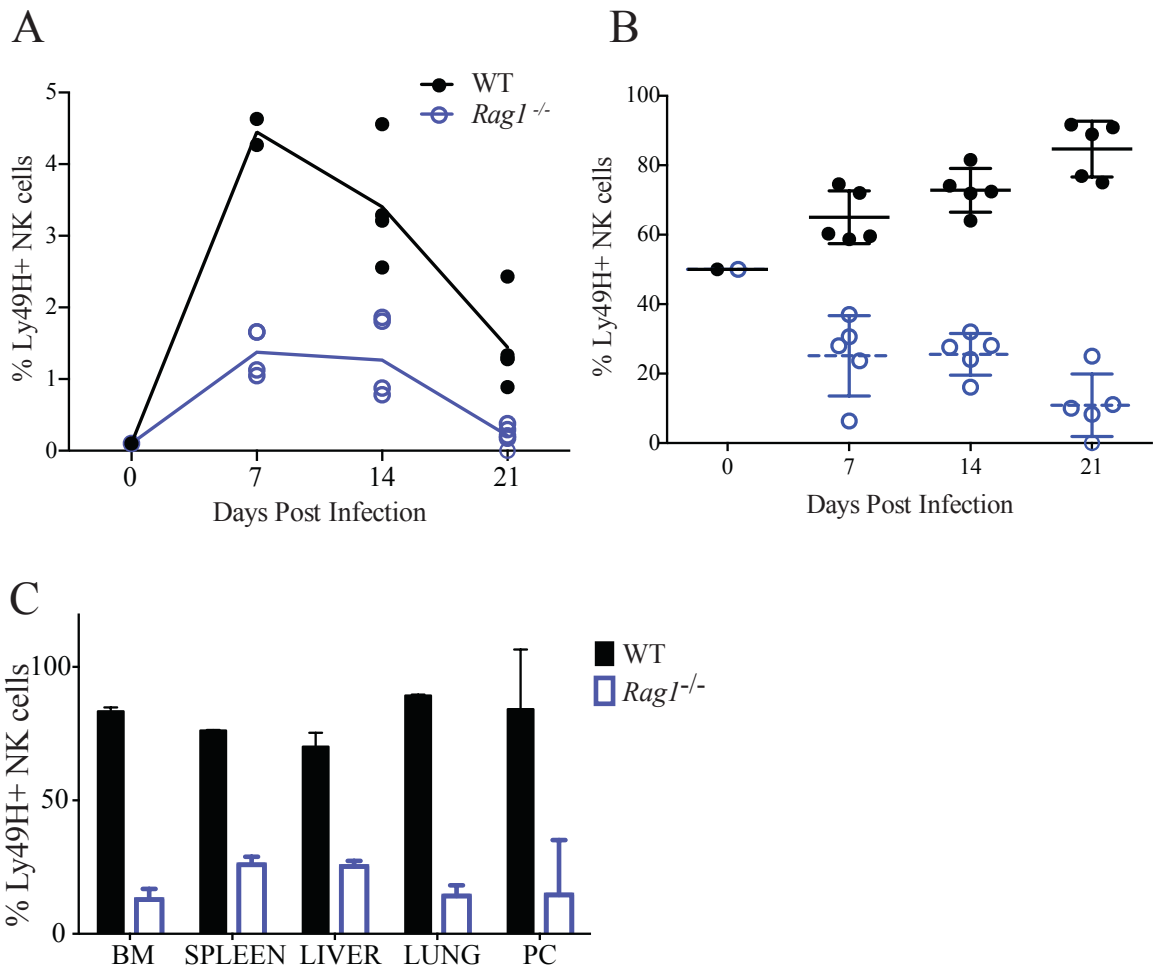

FIGURE 20 | *RAG1*-DEFICIENT NK CELLS RECAPITULATE RAG2-DEFICIENT NK CELL PHENOTYPE.

(A-B) Equal numbers of WT and *Rag1*$^{-/-}$ Ly49H^{+} NK cells were co-transferred into *Ly49h*$^{-/-}$ mice, and following MCMV infection the relative percentages of adoptively transferred populations in peripheral blood are shown for various time points. Data are representative of three independent experiments (n=3-5 mice per time point). **(C)** Graph shows percentage of transferred WT and *Rag1*$^{-/-}$ Ly49H^{+} NK cells in indicated organs at day 10 PI. Error bars show s.e.m. and the graph is representative of three independent experiments with 4-5 mice per group.

deficient NK cells following MCMV infection, regardless of whether the donor NK cells were derived directly from individual WT and *Rag2*$^{-/-}$ mice (Figure 19B-D), or from WT:*Rag2*$^{-/-}$ or WT:*Rag1*$^{-/-}$ mixed bone marrow chimeric mice (i.e. a T and B cell replete environment; Figure 21). In contrast, NK cells from athymic nude behaved like WT NK cells (Figure 22), further underscoring that development in the absence of T cells or AID activity could not solely account for the defect of NK cells from Rag-deficient mice. Adoptive transfer of NK cells from WT:*Rag2*$^{-/-}$ mixed bone marrow chimeric mice into *Rag2*$^{-/-}$ *x Il2rg*$^{-/-}$ hosts (which lack B, T, and NK cells) also demonstrated that *Rag2*$^{-/-}$ NK cells were compromised compared to WT NK cells (Figure 23A). This rules out the possibility that reduced recovery of *Rag2*$^{-/-}$ NK cells is simply due to their being selectively rejected in the Ly49H-deficient hosts. Additionally, we performed an "add back" experiment, where young *Rag2*$^{-/-}$ mice were given T and B cells to reconstitute the lymphopenic compartment. After 8 weeks, *Rag2*$^{-/-}$ NK cells from add back mice were still unable to expand as robustly as WT NK cells following MCMV infection (Figure 23B). Finally, we sorted RFP^{+} and RFP^{-} NK cells from RAG fate-mapping mice and observed that RFP^{+} cells out-competed RFP^{-} cells following MCMV infection when equal numbers were adoptively transferred (Figure 23C). Because neither the mixed bone marrow chimeric setting nor the add back environment were able to rescue the proliferation and survival of *Rag2*$^{-/-}$ NK cells during viral infection, we conclude that RAG-deficiency leads to a cell-intrinsic defect in NK cells. Moreover, we confirmed that mature NK cells do not re-express *Rag* during viral infection (using the *RAG*^{GFP} reporter mice; Figure 24), consistent with the interpretation that RAG activity during NK cell

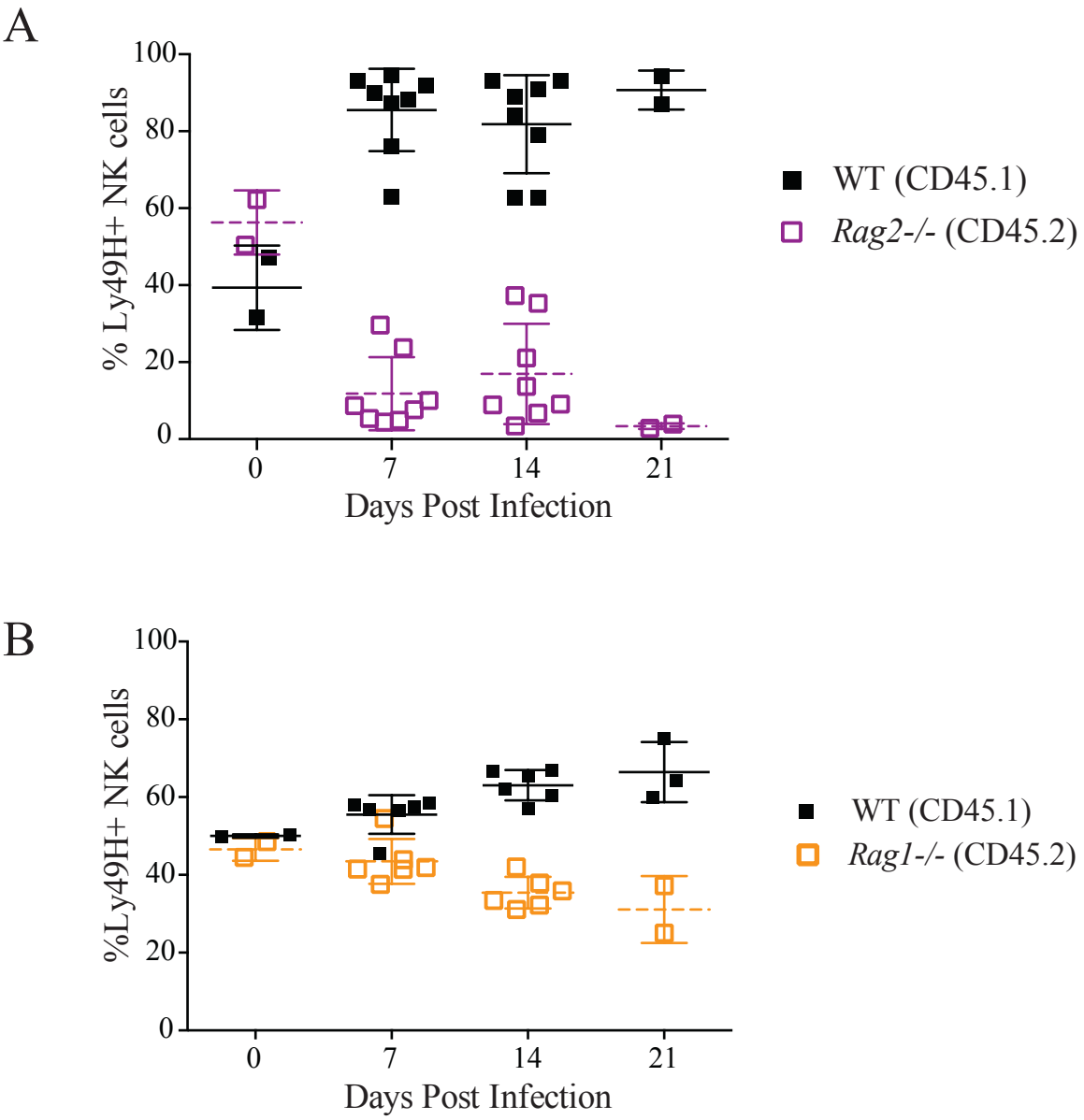

FIGURE 21 | INABILITY OF *RAG*-DEFICIENT NK CELLS TO EXPAND AND PERSIST FOLLOWING MCMV IS RECAPITULATED USING CELLS FROM BONE MARROW CHIMERAS.

(A) Splenic Ly49H^{+} NK cells from mixed WT:*Rag2*$^{-/-}$were adoptively transferred and percentages of donor populations are shown at various time points after MCMV infection. Data are representative of three independent experiments each (with 3-7 mice per time point). **(B)** As in (A) using WT:*Rag1*$^{-/-}$ bone marrow chimeric mice.

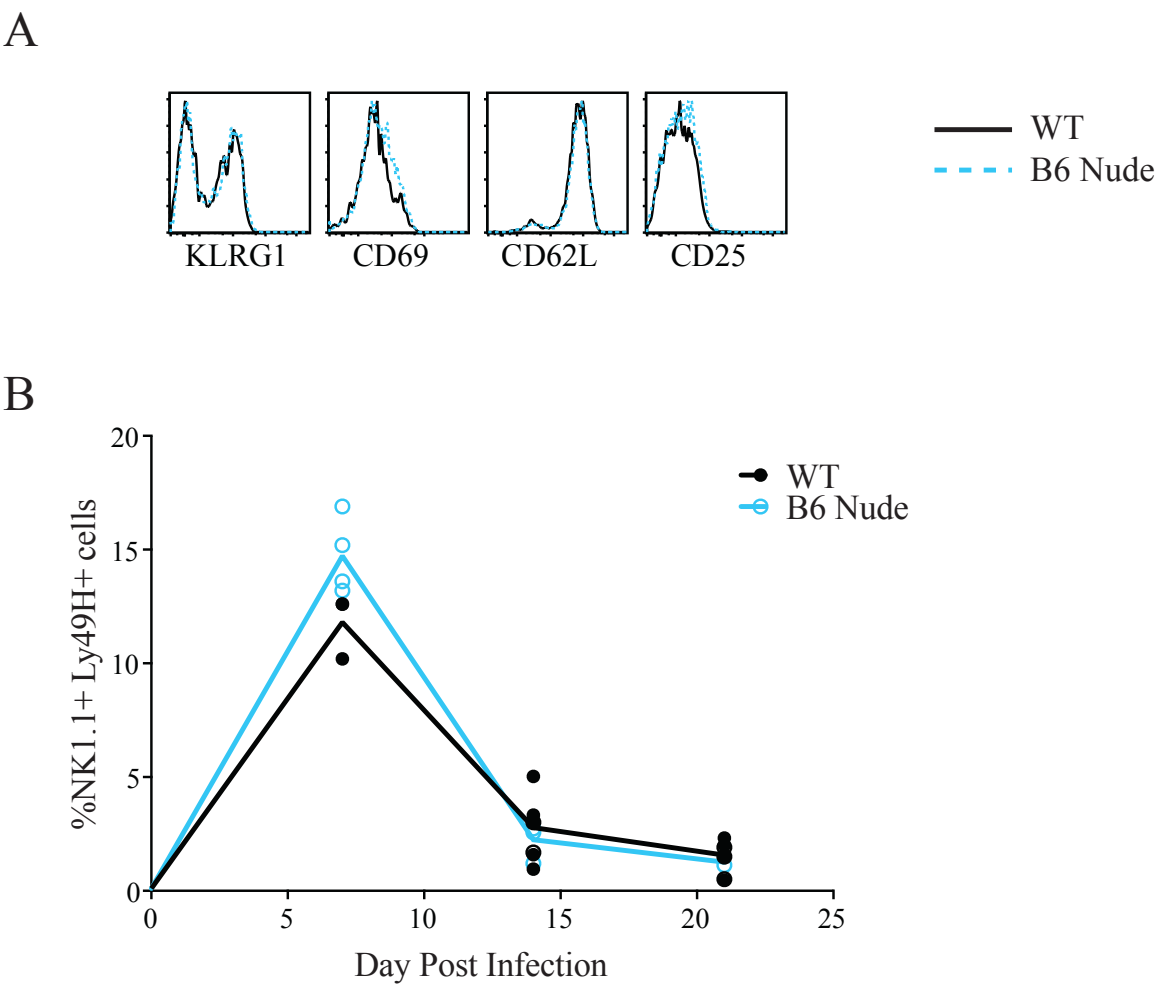

FIGURE 22 | NK CELLS FROM NUDE MICE DO NOT EXHIBIT HYPER-RESPONSIVENESS OR IMPAIRMENT DURING MCMV INFECTION.

(A) Splenic NK cells from WT and Nude mice were analyzed for the activation markers shown. **(B)** WT (CD45.1) and B6 Nude (CD45.2) Ly49H^{+} NK cells from mixed bone marrow chimeric mice were adoptively transferred and percentages of donor populations are shown at various time points after MCMV infection. Results shown are representative of three independent experiments performed with n=3-5 mice, with each data point representing an individual mouse.

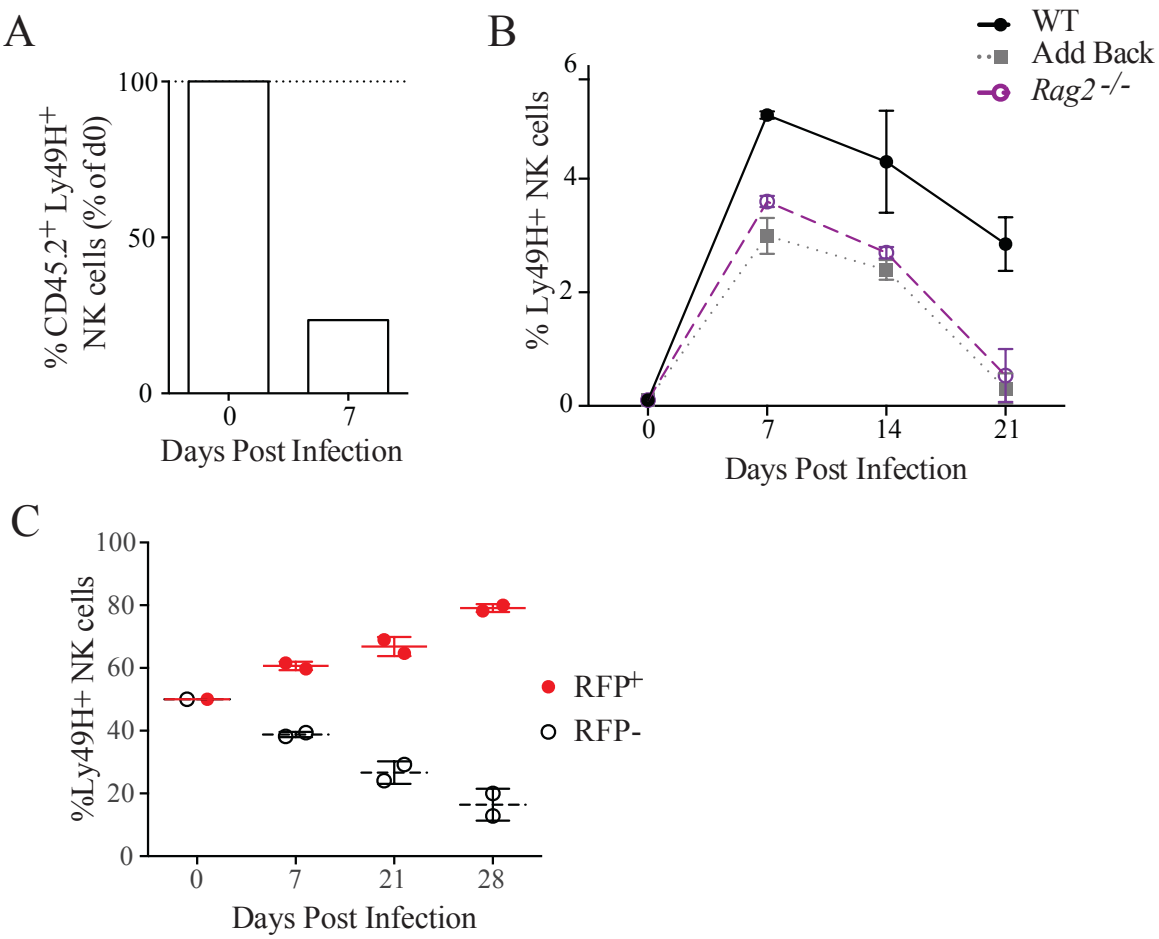

FIGURE 23 | INABILITY OF *RAG*-DEFICIENT NK CELLS TO EXPAND AND PERSIST FOLLOWING MCMV INFECTION IS CELL INTRINSIC.

(A) Splenic Ly49H^{+} NK cells from mixed WT:*Rag2*$^{-/-}$ were adoptively transferred into *Rag2*$^{-/-}$*xIl2rg*$^{-/-}$ hosts and following MCMV, donor NK cell populations were analyzed at day 7 PI. **(B)** Splenic T and B cells were transferred and parked for 8-12 weeks to reconstitute the lymphopenic compartment of young *Rag2*$^{-/-}$ mice (designated "Add back" mice). Ly49H^{+} NK cells from "Add back", WT, and *Rag2*$^{-/-}$ mice were adoptively transferred, and NK cell percentages in peripheral blood at various time points following MCMV infection are shown**.** Results are representative of three independent experiments (n=3-5 mice). **(C)** RFP^{+} and RFP^{-} NK cells were sorted from RAG fate-mapping mice and equal numbers adoptively transferred into *Ly49h*$^{-/-}$ mice. Percentages of donor populations are shown at various time points after MCMV infection. Data are representative of two independent experiments (with 2 mice per time point).

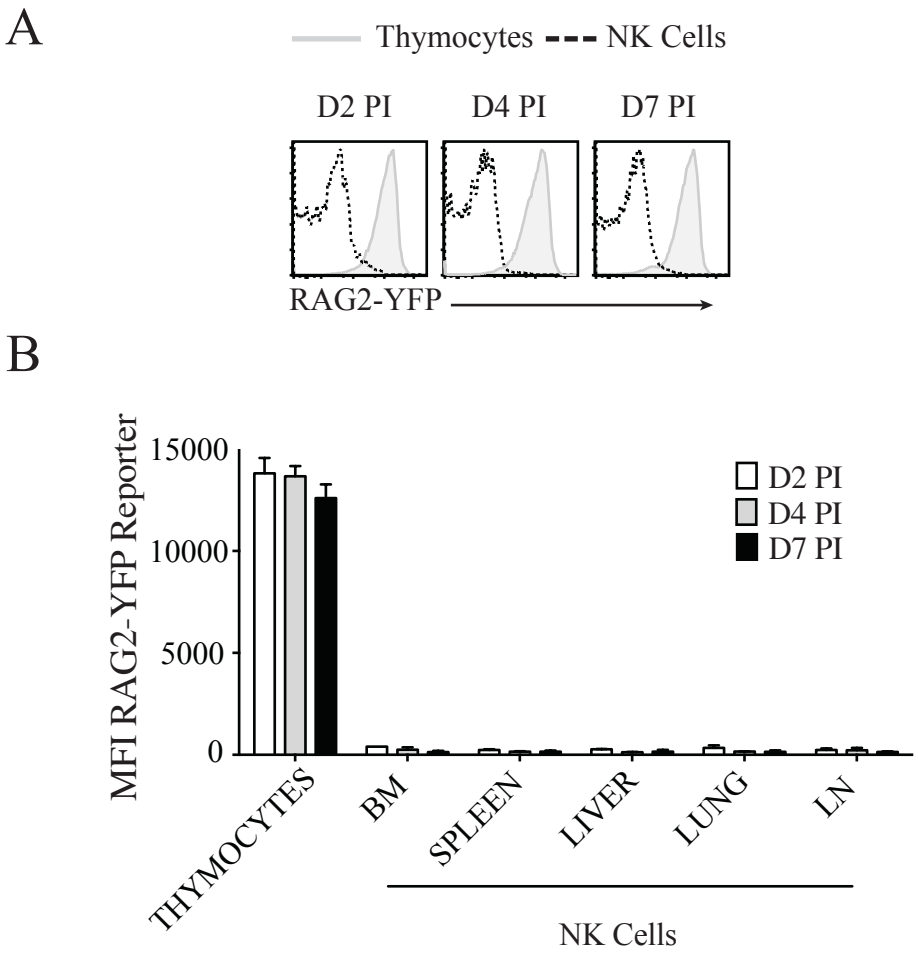

FIGURE 24 | RAG IS NOT RE-EXPRESSED POST-MCMV INFECTION.

(A-B) NK cells from various organs of *Rag2*^{GFP} reporter mice were analyzed for GFP expression at day 2, 4, and 7 after MCMV infection and compared to thymocytes. Representative histograms (A) and quantification (B) are shown. Error bars show s.e.m. and results are representative of three independent experiments (n=3-5 mice).

ontogeny initiates a program of enhanced cellular fitness that influences the peripheral response of mature NK cells to viral infection.

Impaired survival of RAG-deficient NK cells

We next investigated whether the impaired expansion of *Rag2*$^{-/-}$ NK cells during MCMV infection was due to diminished proliferation or impaired survival. When WT and *Rag2*$^{-/-}$ NK cells were labeled with CFSE prior to adoptive transfer and viral infection, similar rates and number of cell division were observed at days 3 and 5 PI (Figure 25A), indicating that *Rag2*$^{-/-}$ NK cells can proliferate normally in response to viral challenge. In contrast, *Rag2*$^{-/-}$ NK cells were significantly more susceptible to apoptosis at steady-state and following MCMV infection, as demonstrated by the significantly higher population staining positive for FLICA, a membrane-permeable probe retained in cells containing active caspases (Figure 25B and data not shown). Furthermore, when NK cells from WT:*Rag2*$^{-/-}$ chimeric mice were pulsed with BrdU and chased to examine the rate of decay of labeled cells, the BrdU-labeled *Rag2*$^{-/-}$ NK cells declined in number more rapidly than WT NK cells (Figure 25C). Together, these findings suggest that RAG expression during NK cell development potentiates the optimal survival of peripheral NK cells.

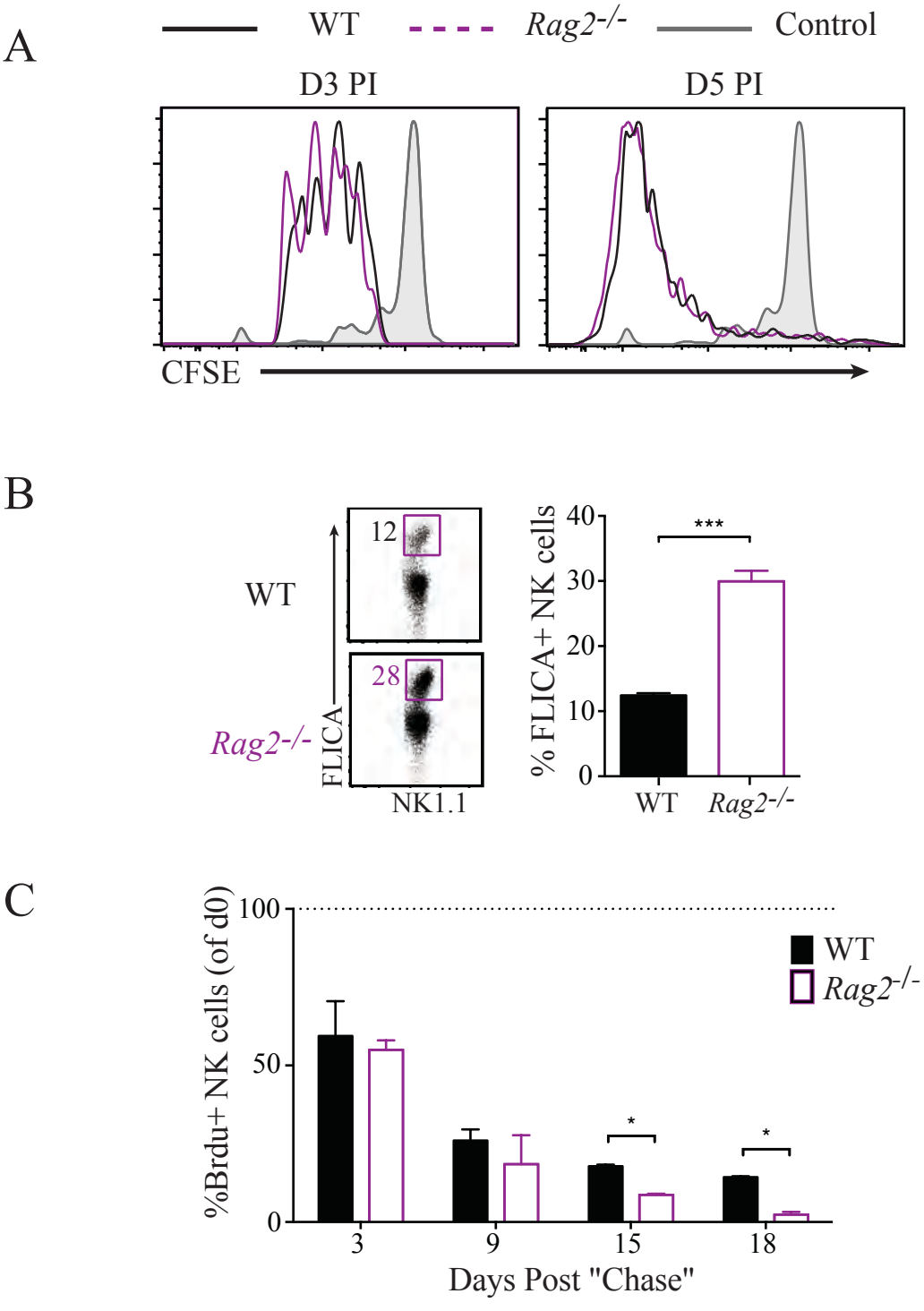

FIGURE 25 | HIGHER RATE OF APOPTOSIS UNDERLIES *RAG*-DEFICIENT NK CELL SURVIVAL DEFECT.

(A) Equal numbers of CFSE-labeled WT (CD45.1) and *Rag2*$^{-/-}$ (CD45.2) Ly49H^{+} NK cells were adoptively transferred to *Ly49h*$^{-/-}$ mice prior to MCMV infection. Histograms show CFSE dilution in the transferred Ly49H^{+} NK cells on days 3 and 5 PI. CFSE-labeled NK cells from uninfected control mice are shown in gray. Data are representative of three independent experiments (n=3-5 mice). **(B)** Representative plots and graph show FLICA staining (for pan-caspase activation) in splenic Ly49H^{+} NK cells from uninfected. Error bars show s.e.m. and data are representative of three independent experiments (n=3-5 mice). *$p < 0.05$; ***$p < 0.0005$. **(C)** *Rag2*$^{-/-}$:WT mixed bone marrow chimeras were pulsed with BrdU (0.8mg/ml) drinking water, and decrease in percent BrdU^{+} NK cells at days after "chase" with normal water was determined. Day 0 marks when mice removed from BrdU water and placed on normal water. Error bars show s.e.m. and data are representative of three independent experiments (n=3-5 mice). *$p < 0.05$.

*Impaired survival not due to changes in Bcl-2 family members**

During T cell memory formation, B-cell lymphoma 2 (Bcl-2) family proteins such as Bcl-2 and Bim play contrasting roles in the survival of antigen-specific effector T cells (Grayson et al., 2000; Hildeman et al., 2002; Prlic and Bevan, 2008). To determine whether lack of survival was due to differences Bcl-2 family members, we examined levels of pro-apoptotic members Bcl-2 and Bcl-XL, and anti-apoptotic member Bim in WT and *Rag2*$^{-/-}$ NK cells following MCMV infection (Figure 26). In uninfected mice, we did not see significant differences in expression of these proteins; however, during viral infection, we find that WT NK cells are slightly lower for all three molecules, compared with RAG2-deficient NK cells (Figure 26). Thus, mechanisms other than the Bcl-2 family of proteins are likely playing a role in maintaining the survival of WT but not RAG-deficient NK cells, but require further elucidation.

*Improved response to B16 melanoma in RAG-deficient mice**

To determine the consequence of RAG depletion on other ailments, we examined the NK cell anti-tumor response to B16 mouse melanoma. B16 mouse melanoma tumor cells do not express MHC class II molecules and express extremely low quantities of MHC class I due to defects in antigen processing machinery (Seliger et al., 2001). Due to its aberrant MHC I expression, B16 tumors should be highly susceptible to NK cell eradication. B16 mouse melanoma is also well suited to study the intricacies of human tumor

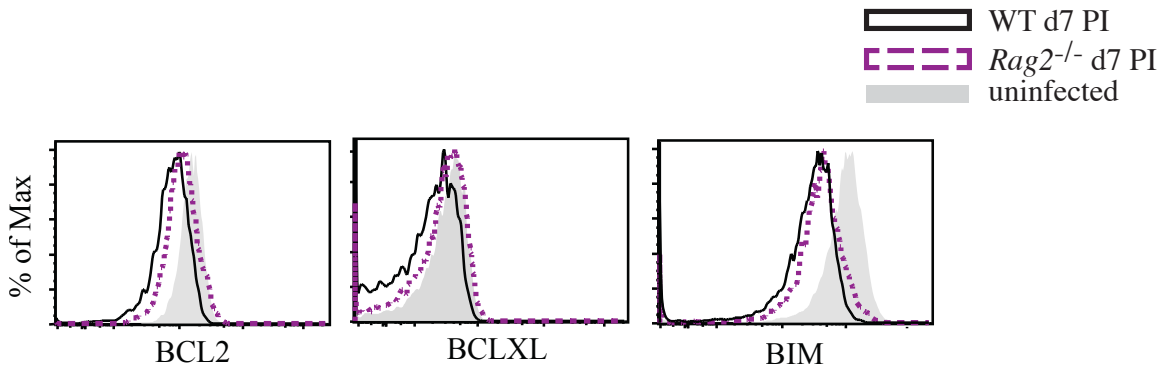

FIGURE 26 | APOPTOSIS IS NOT DUE TO DYSREGULATED BCL-2 FAMILY MEMBERS.

Levels of BCL-2 family members were analyzed at d0 (uninfected, gray) and d7 post-MCMV infection in WT (black) and *Rag2*$^{-/-}$ (purple) splenic NK cells by flow cytometry. Representative histograms from 3 independent experiments shown.

development, progress, and treatment strategies because it is poorly immunogenic (Taniguchi et al., 1985). To analyze the NK cell response against B16, we infected WT or *Rag2*$^{-/-}$ mice with B16 challenge and examined survival, weight loss, and tumor burden (Figure 27). *Rag2*$^{-/-}$ mice responded better than WT mice to tumor challenge (Figure 27A-C), likely due to hyper-responsiveness of NK cells (Figure 15). Further studies of the cytotoxicity of NK cells during B16 tumor challenge are necessary to confirm this. This response was dependent on NK cells, because NK-depleted B6 control mice lost weight rapidly and died before the end of the experiment due to lung and liver colonization (Figure 27A-C).

To carefully dissect the direct role of NK cells in isolation (e.g. without the complexity of contributing T and B cell responses) we used an adoptive transfer system (Figure 27D) to study anti-tumor NK cell responses in a setting where pleiotropic effects can be eliminated. Upon injection into a lymphopenic environment, NK cells have been shown to undergo robust homeostatic proliferation and generate long-lived cells with potent effector function (Sun et al., 2011). In this setting, adoptive transfer of *Rag2*$^{-/-}$ NK cells did not affect overall survival of host mice, compared to WT mice (Figure 27E); however, *Rag2*$^{-/-}$ NK cell transferred mice did lose weight quicker than WT (Figure 27F). This suggests that when there is a constant supply of *Rag2*$^{-/-}$ NK cells (Figure 27B), the enhanced cytotoxicity will protect against B16 tumor burden. However, when the constant source of *Rag2*$^{-/-}$ NK cells is removed (during the adoptive transfer system, Figure 27D) these mice succumb quicker to disease, due to enhanced apoptosis and

turnover of these cells (Figure 27B-C). Experiments to further investigate this mechanism and the physiological role of RAG-expressing NK cells are underway.

*RAG-expression delineates subset heterogeneity similar to CD8+ T cells**

Together, these results suggest RAG-expression during ontogeny delineates subset heterogeneity in peripheral NK cells during viral infection. Interestingly, a paradigm of subset heterogeneity has recently been ascribed to $CD8^+$ T cells following antigen encounter. Following pathogen infection, responding $CD8^+$ T cells generate a heterogeneous effector population consisting of $KLRG1^{hi}$ short-lived effector cells (SLECs) that are more terminally differentiated, and $KLRG1^{lo}$ memory precursor effector cells (MPECs) that can give rise to long-lived memory cells (Kaech and Wherry, 2007). (Figure 28). Given that NK cells are more similar in function and phenotype to effector/memory $CD8^+$ T cells than naïve $CD8^+$ T cells (Sun and Lanier, 2011), we investigated whether a parallel SLEC/MPEC paradigm can explain the heterogeneous peripheral NK cell pool in WT mice, where certain subsets of NK cells (ones that haven't expressed RAG) are more lethal killers in the face of immediate pathogen insult, whereas other subsets (ones that have expressed RAG) contribute to the long-lived memory population that can respond against repeated pathogen exposure. To accomplish this, we compared RNA-seq data (Sue Kaech, unpublished) of memory precursor (MP) and terminal effector (TE) CD8+ T cells to RAG-deficient and WT NK cells (Figure 29). Focusing on a subset of genes that were highly upregulated in either group, we saw MP^{hi} genes subset with WT NK cells, while TE^{hi} genes clustered with RAG-deficient NK cells,

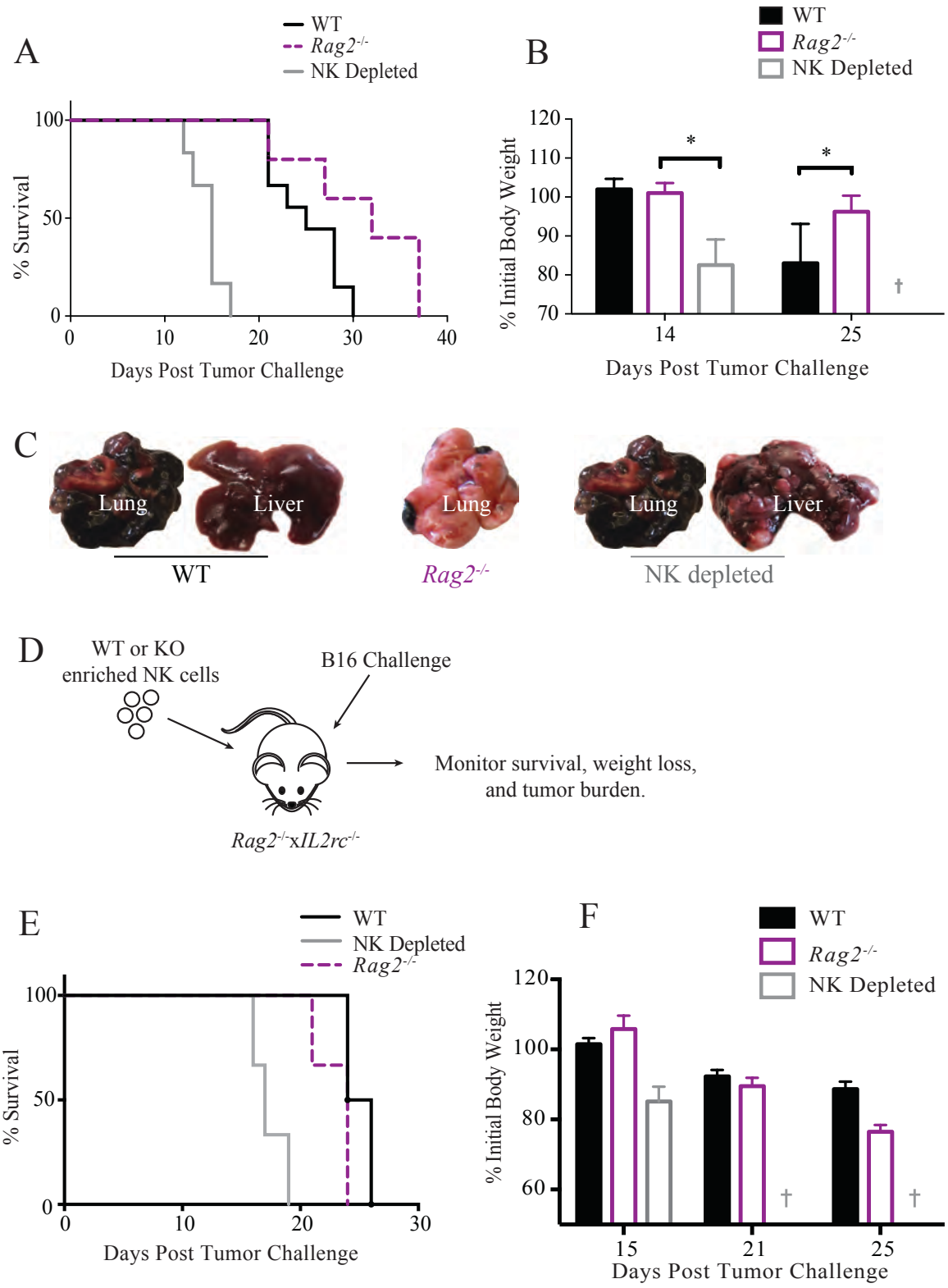

FIGURE 27 | *RAG*-DEFICIENT MICE ARE MORE RESISTANT TO B16 TUMOR CHALLENGE.

(A-C) WT or *Rag2*$^{-/-}$ mice were examined for % survival (A), weight loss (B), and tumor burden at d21 (C) post B16 tumor challenge. WT mice treated with NK depleting antibody (α-PK136) were treated as a control. **(D)** Schematic of adoptive transfer B16 tumor challenge experiment. **(E-F)** Host *Rag2*$^{-/-}$*xIL2rγ*$^{-/-}$ with adoptively transferred WT or *Rag2*$^{-/-}$ NK cells were examined for % survival (E), weight loss (F) post tumor challenge. WT mice treated with NK depleting antibody (α-PK136) were treated as a control.

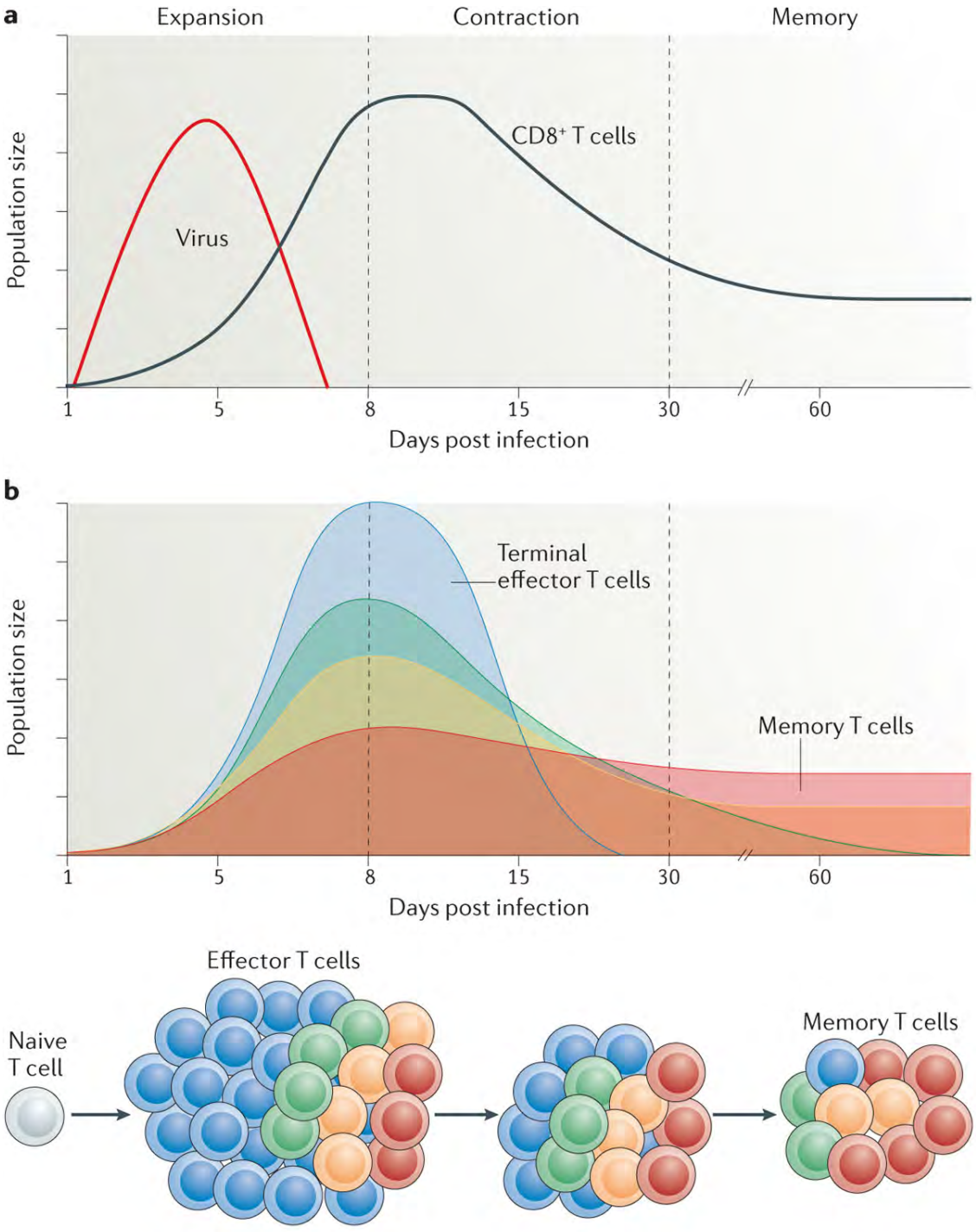

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FIGURE 28 | KINETICS OF A T CELL RESPONSE AND DISTRIBUTION OF MEMORY CELL POTENTIAL.

(A) During an acute viral infection, antigen-specific T cells rapidly proliferate (during the expansion phase) and differentiate into cytotoxic T lymphocytes (CTLs) that mediate viral clearance. Most of these cells die over the next several weeks during the contraction phase of the response. Only a small percentage of effector T cells (5–10%) survive and further develop into functional mature memory $CD8^+$ T cells. **(B)** The pool of effector T cells can be separated into multiple diverse subsets based on differences in gene and protein expression, effector functions, migratory patterns, proliferative capacity and long-term fate. Ultimately, not all effector T cells have equal potential to form memory T cells. Some cell-surface markers correlate with distinct effector and memory T cell fates: terminal effector T cells (shown in blue) are $KLRG1^{hi}$IL-$7R\alpha^{low}CD27^{low}BCL\text{-}2^{low}$, and long-lived memory (and memory precursor) cells (shown in red) are $KLRG1^{low}$IL-$7R\alpha^{hi}CD27^{hi}BCL\text{-}2^{hi}$. However, other T cell subsets with intermediate differentiation states also exist that have mixed phenotypes, longevities and abilities to self-renew, as depicted by the yellow and green populations. Over time, there may also be some interconversion between these subsets.

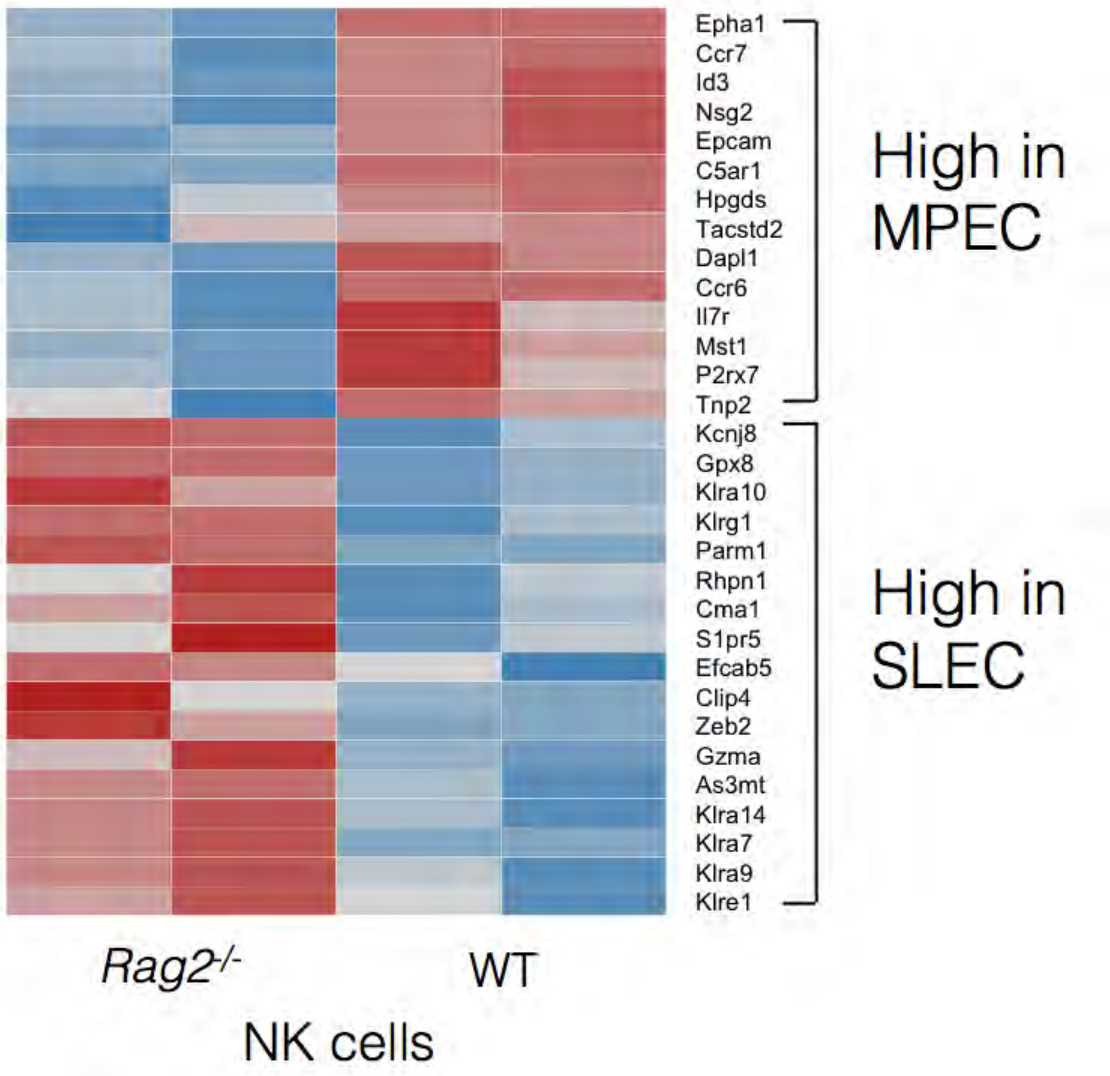

FIGURE 29 | *RAG*-DEFICIENT NK CELLS HAVE A TRANSCRIPTOME REPRESENTATIVE OF TERMINAL EFFECTOR CD8+ T CELLS.

Heatmap of selected differentially expressed genes in Memory Precursor Effector Cells (MPEC) and Short Lived Effector Cells (SLEC) CD8+ T cells and their expression in WT and *Rag2*$^{-/-}$ NK cells.

as hypothesized. These data suggest RAG-mediated DSBs during development may 'imprint' cells (e.g. changes in transcriptome), resulting in subset heterogeneity in the periphery similar to CD8+ T cells.

Discussion

RAG proteins function during lymphocyte development to mediate V(D)J gene rearrangement at the antigen receptor loci, providing the adaptive immune system with a mechanism for assembling and diversifying its antigen receptor gene repertoire. Using RAG fate-mapping and RAG-deficient mice, we were able to distinguish mature lymphocytes that have developed in the presence or absence of RAG-expression and recombination, and analyze their function and survival *in vivo*. Using the RAG fate-mapping mice, NK cells that never expressed RAG during development (RFP^-) were more terminally differentiated (as defined by greater KLRG1 and CD11b expression), and demonstrated a higher degree of cytotoxicity. We further investigated whether RAG influenced *in vivo* NK cell responses by incorporating a well-established viral model of antigen-specific NK cell expansion (Sun et al., 2009). Donor NK cells from RAG1 or RAG2-deficient mice were outcompeted by WT NK cells following adoptive transfer and MCMV infection. NK cells that lacked either RAG or a history of RAG expression were defective in virus-driven expansion and failed to persist due to an increase in apoptosis (Figure 30). These observations represented an interesting dichotomy whereby the most cytotoxic cells (RAG-deficient) had the least capacity to survive, suggesting an important physiological kill-switch to mitigate cytokine storm post-infection.

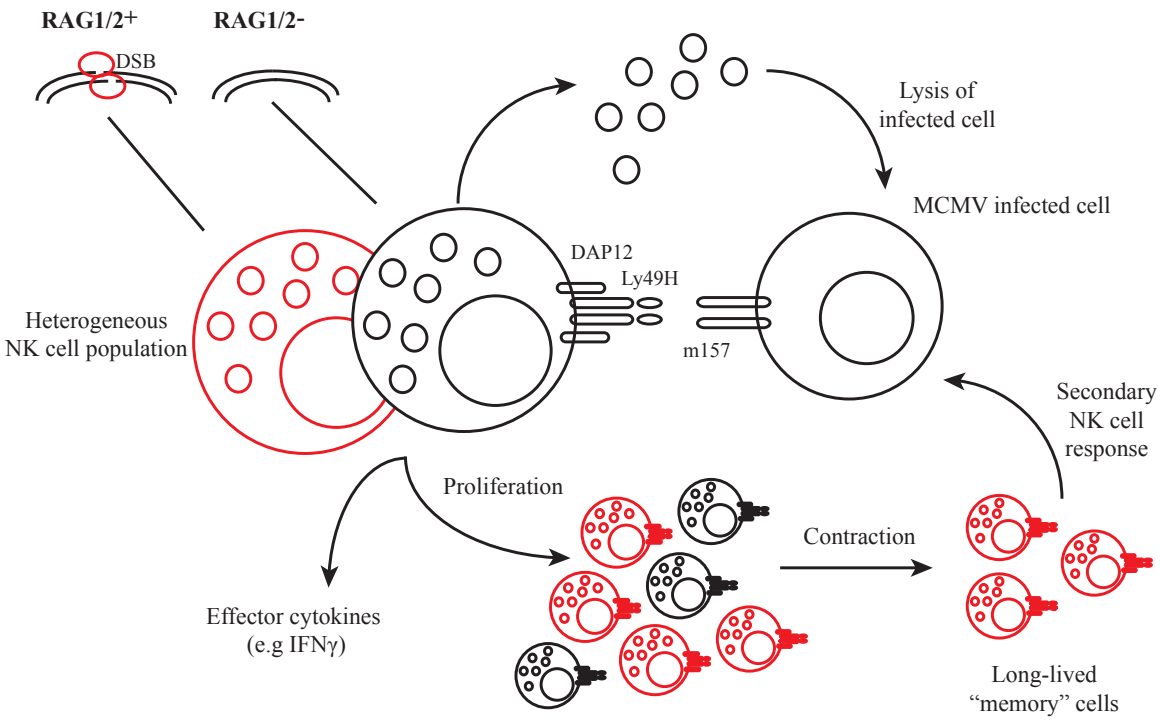

FIGURE 30 | RAG EXPRESSION DURING ONTOGENY DEFINES FUNCITONAL SUBSETS OF NK CELLS IN THE PERIPHERY IN THE PRESENCE OF MCMV INFECTION.

NK cells from B6 mice expressing the activating Ly49H receptor will recognize MCMV-encoded m157 on infected cells, leading to activation and clonal proliferation of antigen-specific NK cells. History of RAG expression marks a subset of NK cells (red) that have enhanced survival following rapid proliferation. After the NK cell expansion phase and viral control, effector Ly49H^{+} NK cells undergo a contraction phase resulting in long-lived memory NK cells, where only the NK cells that previously expressed RAG remain. These memory NK cells are able to mount a recall response when virus is re-encountered.

Another group (Pilbeam et al., 2008) utilized a transgenic mouse recombination reporter system, whereby recombinase activity is indicated by the permanent expression of violet light-excited (VEX) fluorescence (Borghesi and Gerstein, 2004), to mark NK cells that have undergone recombination events. They further analyzed the functional consequences of recombination (VEX+) in NK cells. This group saw ~50% of CLP in bone marrow are VEX+ and 20-30% of peripheral NK cells express the reporter, in agreement with our observations in RAG 'fate-mapping' mice. These authors did not see significant differences in their recombination-positive versus recombination-negative NK cells, however they did note that V(D)J recombination does permanently mark functional subsets of NK cells. Our observations complement their findings, and further demonstrate functional differences within these distinct subsets post-MCMV infection.

Using various different model systems, we showed our observed phenotype was cell-intrinsic, rather than a result of cell-extrinsic factors in RAG-deficient mice (e.g. lack of adaptive immune system). However, a recent study (Kamimura and Lanier, 2015) demonstrated that less terminally differentiated KLRG1$^-$ NK cells (phenotypically similar to WT NK cells) are more likely to represent memory progenitor cells, retaining the ability to generate long-lived memory cells. This paper addresses many cell extrinsic factors that govern these KLRG1$^-$ NK memory precursor populations, implicating both T cells competing for common cytokines, and the microbiota. Thus, the lack of T cells in RAG-deficient mice also contributes to the defect in memory NK cell generation in these animals, and represents an extrinsic mechanism complementary to the cell-intrinsic role

for RAG described in our study. Additional cell-intrinsic and extrinsic factors that dictate memory-precursor NK cell populations remain to be determined.

In addition to evidence supporting NK cell memory formation during MCMV infection, studies have suggested that NK cells are essential for secondary responses in other mouse models of viral infections. Adoptive transfer experiments in mice demonstrate that $CXCR6^{+}$ hepatic NK cells primed with virus-like particles are sufficient and required for protective recall responses against vesicular stomatitis virus (VSV), influenza A, and human immunodeficiency virus (HIV) (Paust et al., 2010). These studies collectively support recall responses of memory NK cells in several models, but are still limited by unknown interactions between NK cell receptors and cognate pathogen-encoded antigens that may be mediating these responses. Further, Yokoyama and colleagues have also found that activation by cytokines, including IL-12, IL-15, and IL-18, alone leads to the generation of NK cells with memory-like properties (Cooper et al., 2009). After overnight stimulation with these cytokines, mouse splenic NK cells exhibited massive *in vivo* proliferation and elevated IFNγ levels upon *ex* vivo stimulation with either cytokines (IL-12 and IL-15) or through engagement of activating receptors for up to three weeks after the transfer. The authors were able to demonstrate that progeny from the stimulated cells could also exhibit similar functionality, suggesting memory-like properties could be passed on to daughter generations (potentially through epigenetic imprinting (Kanno et al., 2012; Weng et al., 2012)). No distinct surface phenotype change has been described to discriminate cytokine-induced memory-like NK cells from their naïve or activated counterparts. Cerwenka and colleagues demonstrated the role of cytokine-induced

memory-like NK cells cooperate with CD4+ T cells to mediate effective antitumor activity in a mouse model of lymphoma [162], suggesting a role for cytokine-induced memory outside of infection. It would be of interest to analyze RAG-mediated subset heterogeneity in models of inflammatory cytokine signaling and hapten-specific induced NK cell memory and determine the environments necessary for the phenotypes observed.

The generation of long-term immunity to infectious disease is dependent on the formation of long-lived memory CD8+ T cells. Compared to naïve T cells, these cells are present in greater number and are poised for rapid response to secondary infection. These cells are maintained through a process of self-renewal driven by IL-15 and IL-7 (Boyman et al., 2007). Compared to effector T cells, memory CD8 T cells are less terminally differentiated, have a high proliferative potential and increased longevity (Joshi et al., 2007). In studies with acute lymphocytic choriomeningitis virus (LCMV) and *listeria monocytogenes* bacterial infection, cells with memory potential (MPECs) were found to be KLRG1^{lo} and IL7R^{hi}, while SLECs were defined as KLRG1^{hi} and IL7R^{lo} (Joshi et al., 2007.). Interestingly, these results correlate with our WT and *Rag2*$^{-/-}$ data, where WT are similar transcriptionally to MPECs and *Rag2*$^{-/-}$ NK cells cluster to SLECs (Figure 29). Moreover, RAG DSBs have been shown to initiate expression of lymphocyte specific programs, including upregulation of IL-7R in pre-B cells (Bredemeyer et al., 2008). Activation of IL-7R in these cells leads to the activation of STAT5, leading to upregulation of anti-apoptotic pathways and pro-proliferation genes (Corfe and Paige, 2012). Further examination of the transcriptional changes resulting from RAG expression

during ontogeny would provide insight to whether the functional heterogeneity seen post MCMV in NK cells is parallel to the MPEC and SLEC model identified in CD8+ T cells.

Interestingly, *Rag2*$^{-/-}$ mice were more protected against B16 melanoma compared to WT mice; however, a different result was observed when *Rag2*$^{-/-}$ or WT NK cells were transferred into NK cell-deficient mice followed by tumor challenge (Figure 27D). Upon injection into a lymphopenic environment, NK cells have been shown to undergo homeostatic proliferation and generate long-lived cells with potent effector function (Sun et al., 2011). Therefore, upon adoptive transfer into a lymphopenic host the *Rag2*$^{-/-}$ NK cells may undergo apoptosis due to their impaired survival post robust proliferation (Figure 27F), never making it to the site of tumor challenge. In the adoptive transfer model, the NK cells cannot be replenished after undergoing apoptosis; however, in *Rag2*$^{-/-}$ hosts, there is a constant supply of hyper-responsive NK cells. Another explanation of these differing observations is *Rag2*-/- mice (Figure 27A-C) lack regulatory T cells, a subset of CD4+ T cells that produce suppressive cytokines (e.g. IL-10 and TGFβ), in the tumor microenvironment. While regulatory T cells do not affect NK cell responses directly (Gasteiger et al., 2013), the role of IL-10 and TGFβ on the function of NK cells is unknown (and currently under investigation in the lab). Further, there may be an independent mechanism whereby regulatory T cells in the tumor microenvironment affect the recruitment or cytotoxicity of NK cells to the site of tumor challenge or myeloid derived suppressor cells (MDSCs) often found in tumor microenvironment and known to affect NK cells (Li et al., 2009). Further studies are necessary to determine the physiological roles of RAG-expressing and RAG-deficient NK cells during MCMV and

tumor challenge. Unlike the tumor microenvironment, DCs produce IL-12 and IL-18 during viral infection that enhances the NK cell cytotoxic IFNγ response through JAK/STAT4 signaling. The role of these differentiated cytokine milieus on RAG-expressing or RAG-deficient NK cells during infection and tumor challenge remains to be investigated.

Based on our observations, lack of RAG expression during ontogeny results in higher turnover of NK cells and apoptosis at steady state. However, the mechanism for this enhanced turnover and decreased survival is unknown. NK cells sense IL-2 and IL-15 at steady state through the common γ-chain (IL2*r*γ), which is essential for NK cell development (Kasahara et al., 2004 104). Interestingly, RAG-mediated double strand breaks induce expression of IL2rγ (Kovanen and Leonard, 2004; Willerford et al., 1995). Therefore, NK cells lacking RAG expression may have aberrant expression of IL2rγ receptor resulting in diminished cytokine sensing and survival of these cells at steady state. The effect of RAG expression on developmental pathways and changes in transcription will be further addressed in Chapter 5. Further, our data are consistent with published reports showing downregulation of Bcl-2 family proteins in both effector CD8+ T cells and NK cells during viral infection (Beaulieu et al., 2014; Dunkle et al., 2013; Grayson et al., 2000). Bcl-2 levels are known to inversely correlate with proliferation in effector T cells, such that the most proliferative cells are thought to have a diminished capacity to survive in the long-term. This is in line with our current findings where we find larger numbers of WT NK cells than RAG-deficient NK cells at day 7 after MCMV infection. However, the death of highly proliferative, $Bcl2^{lo}$ T cells is also

known to dictate the contraction phase of effector response, which is not consistent with the greater rate of cell death in the RAG-deficient NK cells. Furthermore, Bim-mediated pro-apoptotic signaling during the contraction phase is required to form a stable pool of memory cells (Min-Oo et al., 2014). Thus, mechanisms other than the Bcl2 family of proteins are likely playing a role in maintaining the survival of WT but not RAG-deficient NK cells.

To summarize, we observed that NK cells lacking RAG (or RAG activity) or WT NK cells lacking a history of RAG expression are more terminally differentiated and highly cytolytic, but these short-lived effector cells are characterized by greater apoptosis. In contrast, WT NK cells with a history of RAG expression are less terminally differentiated and cytotoxic, but can generate long-lived memory cells following antigen-specific proliferation, characterized by increased survival. Additional studies will be required to determine whether NK cells that have previously expressed RAG become functionally specialized through upregulation of lymphoid-related transcription factors, resulting in the cellular "fitness" observed following periods of robust proliferation. To determine the mechanism resulting in this functional dichotomy between NK cell populations, we analyzed the mechanism of RAG function – focusing on RAG-generated DNA breaks and the role of RAG in maintain genomic stability. These results are detailed in the following chapter.

CHAPTER 4: RESULTS II

Genomic stability induced by RAG expression in NK cells during ontogeny

Introduction

The V(D)J recombination reaction is initiated when RAG recombinase introduces DNA DSBs within antigen receptor loci (Fugmann et al., 2000b). Therefore, this process possesses great potential for genomic instability, since DSBs can initiate chromosomal translocations (Zhang et al., 2010). To combat this, RAG proteins are restricted to the G1 phase of cell cycle by phosphorylation and degradation of RAG2 (Desiderio et al., 1996)and the broken ends formed during the cleavage stage of recombination are quickly shuttled into the NHEJ DDR pathway. The core NHEJ factors Artemis, DNAPK, KU70 and Ku80 are necessary for forming a functional antigen receptor (Helmink and Sleckman, 2012a). Further ATM promotes the stability of the broken ends in a PCC until they can be joined (Bredemeyer et al., 2006). High efficiency in this process is essential to maintain genomic integrity in developing lymphocytes.

DNA-PKcs (DNA-PK catalytic subunit) (Lees-Miller, 2008; Smith and Jackson, 1999) and ATM (Helmink and Sleckman, 2012a; Shiloh, 2003) are members of the PI3K-like family of serine threonine kinases essential for activating downstream effectors of the NHEJ damage response post activation. Endonuclease activity of Artemis (Lieber, 2010)

is dependent on DNA-PKcs (Goodarzi et al., 2006; Smith and Jackson, 1999) and is essential for the completion of the V(D)J reaction. Deficiency of DNA-PKcs in mice leads to severe combined immunodeficiency (SCID) due to the requirement for DNA-PK in receptor antigen formation (Helmink and Sleckman, 2012a; Rooney et al., 2002). In humans, DNA-PKcs null mutations have not been identified in patients (van der Burg et al., 2009). Humans and mice deficient in ATM develop ataxia-telangiectasia (A-T), which is a multisystem disease comprising of cerebellar ataxia, genomic instability, and predisposition to developing lymphoid tumors with translocations involving antigen receptor loci (Barlow et al., 1996; Elson et al., 1996; Lavin and Shiloh, 1997; Xu et al., 1996). Manifestation of disease, and the observation that mice lacking these repair enzymes are sensitive to ionization radiation, suggests an essential role for these proteins in maintaining genomic stability (Bednarski and Sleckman, 2012).

In response to DNA DSBs, ATM and DNA-PKcs phosphorylate hundreds of targets necessary for the downstream activation of DDR pathways and the initiation of apoptosis in cells that fail to repair DNA breaks (Bednarski and Sleckman, 2012; Matsuoka et al., 2007; Rouse and Jackson, 2002; Zhou and Elledge, 2000). Specifically, ATM phosphorylates the CHK2 kinase leading to the phosphorylation of Cdc25a promoting cell cycle arrest (Reinhardt and Yaffe, 2009). ATM and DNA-PKcs also phosphorylate the histone protein H2AX in chromatin flanking DNA DSBs (Fernandez-Capetillo et al., 2004; Rogakou et al., 1998a). Phosphorylated H2AX (γH2AX) at the site of DSB (known as foci) recruits DNA damage response proteins to the site of broken DNA (Fernandez-Capetillo et al., 2004). Clearance of phosphorylation signifies repair of the DSB. Further,

these kinases activate a multitude of downstream effector molecules that regulate transcriptional programs to balance pro-apoptotic (or death) and pro-survival pathways within rearranging cells (Bednarski and Sleckman, 2012).

Our previous data (Chapter 3) demonstrates that RAG expression during development mark functionally distinct subsets of mature NK cells in the periphery, where NK cells that have a history of RAG expression seem to have a survival advantage compared to those that lack RAG expression following proliferation. Here we examine the role for the RAG recombinase and the DDR beyond their canonical pathways. We demonstrate that RAG-deficient NK cells are more prone to apoptosis at steady state, contain a greater amount of phosphorylated γ-H2AX indicative of genomic instability, and are less efficient at processing or repairing genomic breaks during DNA damage. Further, RAG-deficient NK cells have reduced levels of DNA damage response mediators such as DNA-PKcs, Ku80, and ATM, and these repair enzymes were also shown to be critical for cell survival following virus infection. These findings identify a surprising and novel role for RAG in the functional specialization of NK cells, which involves conferring cellular fitness in NK cell subsets via endonuclease activity and unknown mechanisms beyond V(D)J recombination.

Results

RAG-deficient NK cells contain more DNA double strand breaks and reduced levels of DNA repair enzymes

To investigate why *Rag2*$^{-/-}$ NK cells are more prone to apoptosis, even at steady-state, we assessed levels of phosphorylated histone H2AX (γ-H2AX) in WT and *Rag2*$^{-/-}$ NK cells as a measure of DNA double-strand breaks (DSBs) and global genomic instability (Fernandez-Capetillo et al., 2004; Rogakou et al., 2000a; Rogakou et al., 1998b). Notably, *Rag2*$^{-/-}$ NK cells showed higher levels of γ-H2AX compared to WT NK cells by flow cytometry and immunohistochemistry (Figure 31A-B). In eukaryotic cells, small amounts of ionizing radiation (IR) induce DNA DSBs and γ-H2AX foci formation (Rogakou et al., 1998b), followed by DNA DSB repair and subsequent removal of the γ-H2AX mark. Thus, to compare the DSB repair response of WT and *Rag2*$^{-/-}$ NK cells, we measured γ-H2AX levels at various time points after exposure to radiation (10 Gy). As expected, WT NK cells upregulated γ-H2AX within 2 hours of radiation treatment, followed by clearance of γ-H2AX foci within 24 hours (Figure 31C). In contrast, *Rag2*$^{-/-}$ NK cells showed a delay in upregulation of γ-H2AX and a marked defect in their ability to clear γ-H2AX foci, indicating a defect in processing or repair of DSBs (Figure 31C, dashed line). These findings suggest that NK cells lacking RAG activity during ontogeny both accumulate evidence of DNA damage at steady state and have difficulty mediating a DNA repair response following the acute introduction of DNA damage.

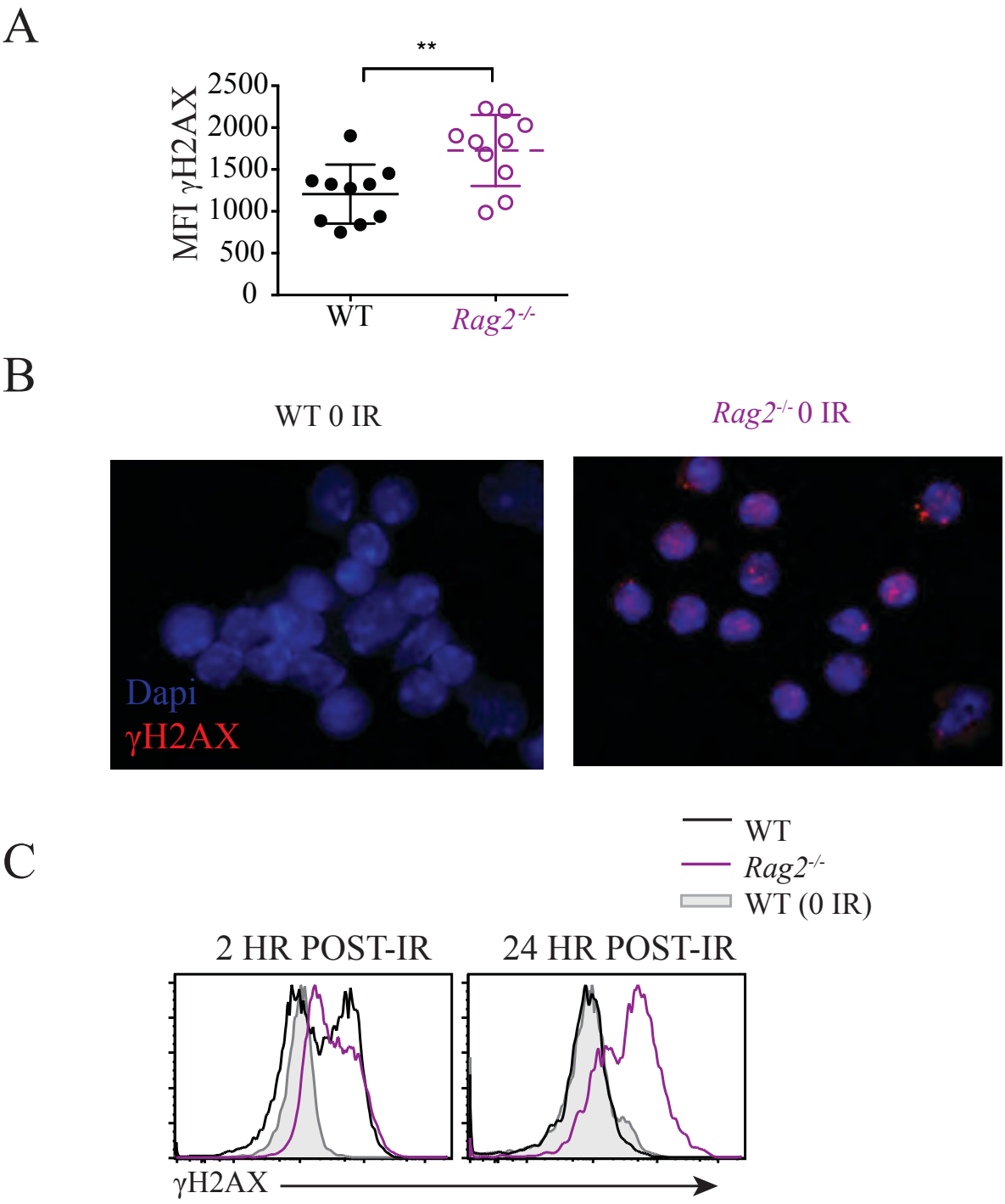

FIGURE 31 | *RAG*-DEFICIENCY DURING ONTOGENY PRODUCES NK CELLS WITH HIGHER AMOUNT OF DSBS AND INEFFICIENT REPAIR.

(A) Mean fluorescence intensity (MFI) of γ-H2AX on splenic NK cells from WT and *Rag2*$^{-/-}$ mice. Data are representative of three independent experiments (n=5-10 mice), with each data point representing an individual mouse. **p < 0.005. **(B)** Representative images of γH2AX immunohistochemistry (IHC) at steady state of WT (left) and Rag-deficient (B) sorted-NK cells. **(C)** WT and *Rag2*$^{-/-}$ NK cells from mixed bone marrow chimeric mice were irradiated (IR, 10 Gy) and phosphorylation of H2AX was analyzed by flow cytometry at various time points post-IR. Representative histograms show irradiated WT (solid) and *Rag2*$^{-/-}$ (dashed) NK cells compared to unirradiated cells (0 IR, gray).

Because RAG functions to generate DNA DSBs during V(D)J recombination in developing T and B cells (Chen et al., 2000; Helmink and Sleckman, 2012b), it seemed counter-intuitive that RAG-deficiency would result in elevated levels of basal DSBs in resting NK cells. However, one important consequence of RAG-mediated DSBs in lymphocytes is the activation of DNA repair and the induction of a broad program of changes in gene expression that includes factors involved in the DNA damage response (Bredemeyer et al., 2008; Helmink and Sleckman, 2012b). Thus, we hypothesized that loss of RAG in developing NK cells could result in diminished expression or activity of DNA damage repair enzymes, and thereby contribute to genomic instability when DSBs arise as a result of proliferation or cellular stress. In support of this hypothesis, *Rag2*$^{-/-}$ NK cells were found to have lower transcript levels of DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ataxia telangiectasia mutated (ATM), Ku80 (Xrcc5), and Chek2 (an essential protein downstream of ATM (Helmink and Sleckman, 2012b)), as compared to WT NK cells (Figure 32A). This was also seen at the protein level using flow cytometry (Figure 32B*). Because ATM and DNA-PKcs have serine/threonine kinase activity and are thought to phosphorylate H2AX (Chen et al., 2000; Fernandez-Capetillo et al., 2004; Helmink and Sleckman, 2012b; Rogakou et al., 1998b), a decrease in their levels might explain the delay in upregulation of γ-H2AX in irradiated *Rag2*$^{-/-}$ NK cells. Decreased levels of DNA-PKcs and Ku80 may also explain the higher rate of apoptosis in *Rag2*$^{-/-}$ NK cells compared to WT NK cells (Figure 32B), as both DNA-PKcs and Ku80 are critical for NHEJ (Bredemeyer et al., 2008; Helmink and Sleckman, 2012b), and reduced levels could reduce DSB repair and maintenance of DNA integrity.

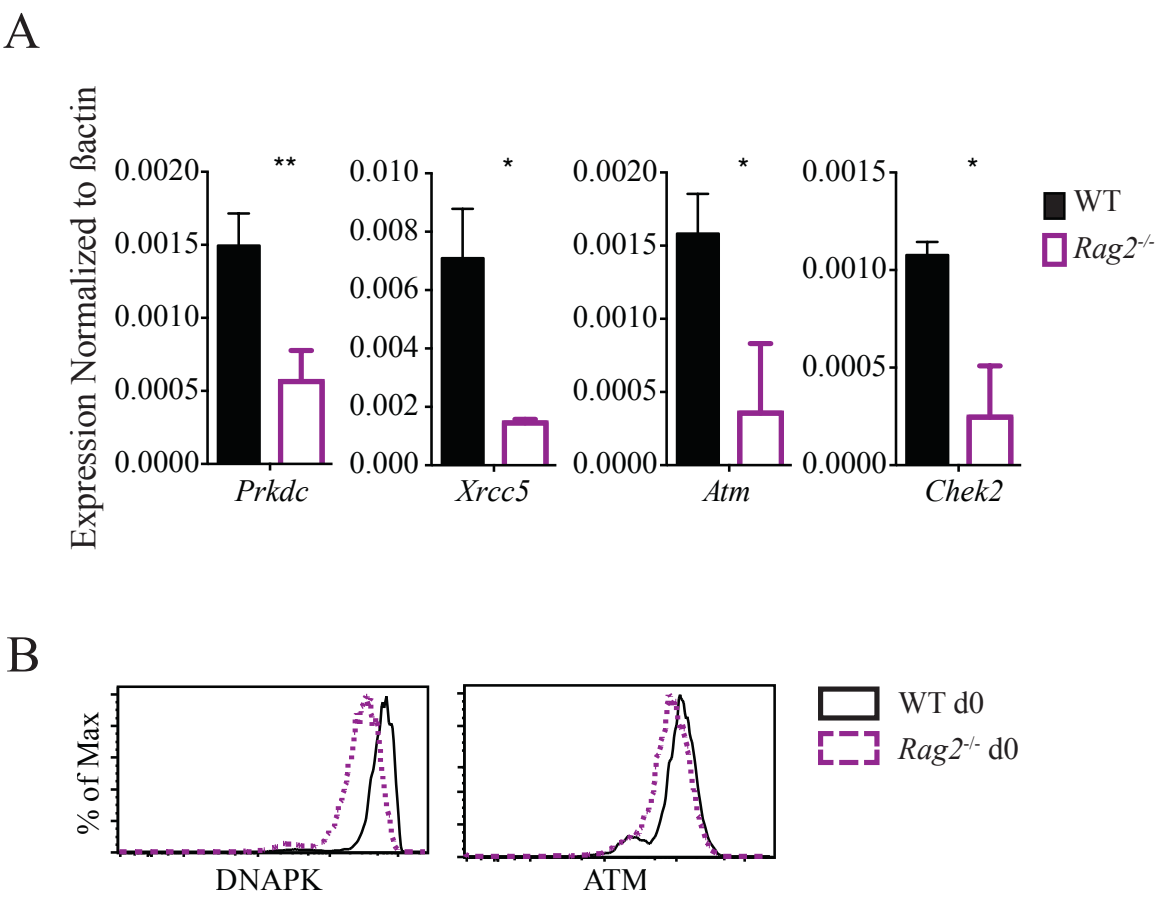

FIGURE 32 | *RAG*-DEFICIENCY DURING ONTOGENY PRODUCES NK CELLS WITH REDUCED DNA BREAK REPAIR.

(A) *Prkdc, Xrcc5, Atm,* and *Chek2* mRNA levels were quantified by qRT-PCR in sorted WT and *Rag2*$^{-/-}$ NK cells. Gene expression levels are normalized to β-actin. Results shown are representative of three independent experiments. **(B)** Protein levels of DNA PK (left) and ATM (right) was analyzed by flow cytometry at steady state. Representative histograms show steady state WT (solid) and *Rag2*-/- (dashed) NK cells.

*Decreased DDR transcript expression is not a hallmark of terminal differentiation**

While decreased DDR transcript expression may be a result of lack of RAG expression during ontogeny in *Rag2*$^{-/-}$ NK cells, it is possible that the reduction in DDR might be a hallmark of these cells being more terminally differentiated. Consequently, maintenance of genome integrity may be less important in these more mature cells. To address this, we examined the genomic integrity of NK cells at different maturation states using γH2AX staining and saw no differences in γH2AX expression in immature (CD27+ CD11b-), intermediate (CD27+ CD11b+) or mature (CD27- CD11b+KLRG^{hi}) NK cells (data not shown). Second, we sorted KLRG1^{hi} and KLRG1^{lo} cells from WT mice and analyzed transcript levels of DDR genes by qPCR (Figure 33*). These data show that terminally differentiated KLRG1^{hi} cells do not have significant differences in DDR genes compared to KLRG1^{lo} NK cells. Together, these lines of evidence suggest that it is not simply terminal differentiation that accounts for the phenotypes observed in RAG-deficient versus WT NK cells, and terminal differentiation alone does not decrease the ability of cells to repair DNA breaks.

Diminished cellular fitness in hyper-responsive NK cells from SCID mice

Our data indicate that RAG-deficiency results in diminished DNA-PKcs in NK and CD8^{+} T cells. To ascertain whether these diminished DNA-PKcs levels might fully or partially

account for the survival defect of *Rag2*$^{-/-}$ NK cells during viral infection, we analyzed NK cells from severe combine immunodeficiency (SCID) mice, which harbor a naturally-occurring nonsense mutation in DNA-PKcs (Blunt et al., 1996; Bosma et al., 1983; Fulop and Phillips, 1990). Although DNA-PKcs is required for V(D)J recombination and therefore T and B cell development, SCID animals are similar to RAG-deficient animals in that they harbor normal NK cell numbers (Figure 34A). Indeed, like RAG-deficient NK cells, we found that NK cells from SCID mice exhibited an activated phenotype (Figure 34B) and increased cytotoxic potential (Figure 34C), but diminished survival following MCMV infection compared to WT NK cells (Figure 35A). SCID NK cells could not be recovered in any organs by 3 weeks PI, in contrast to the robust generation of long-lived NK cells from WT mice (Figure 35B). In addition, the SCID NK cells had higher γ-H2AX levels compared to WT NK cells (Figure 36A), but were delayed in γ-H2AX upregulation and clearance following IR treatment (Figure 36B). SCID NK cells also showed diminished transcript levels of required NHEJ proteins Ku80 (*Xrcc5*), Artemis, and Chk2 (Figure 36C*). Because our result in mice with the spontaneous SCID mutation mirrors the findings in *Rag1*- and *Rag2*-deficient mice, this alleviates potential complications and unwanted phenotypes from genetic knockout mice generated by incorporation of a neomycin selection marker. Taken together, our data support a model where RAG is required for optimal DNA-PKcs expression in NK cells and that DNA-PKcs, in turn, has an important role in maintaining lymphocyte genomic stability and survival following DNA damage, such as occurs during rapid proliferation.

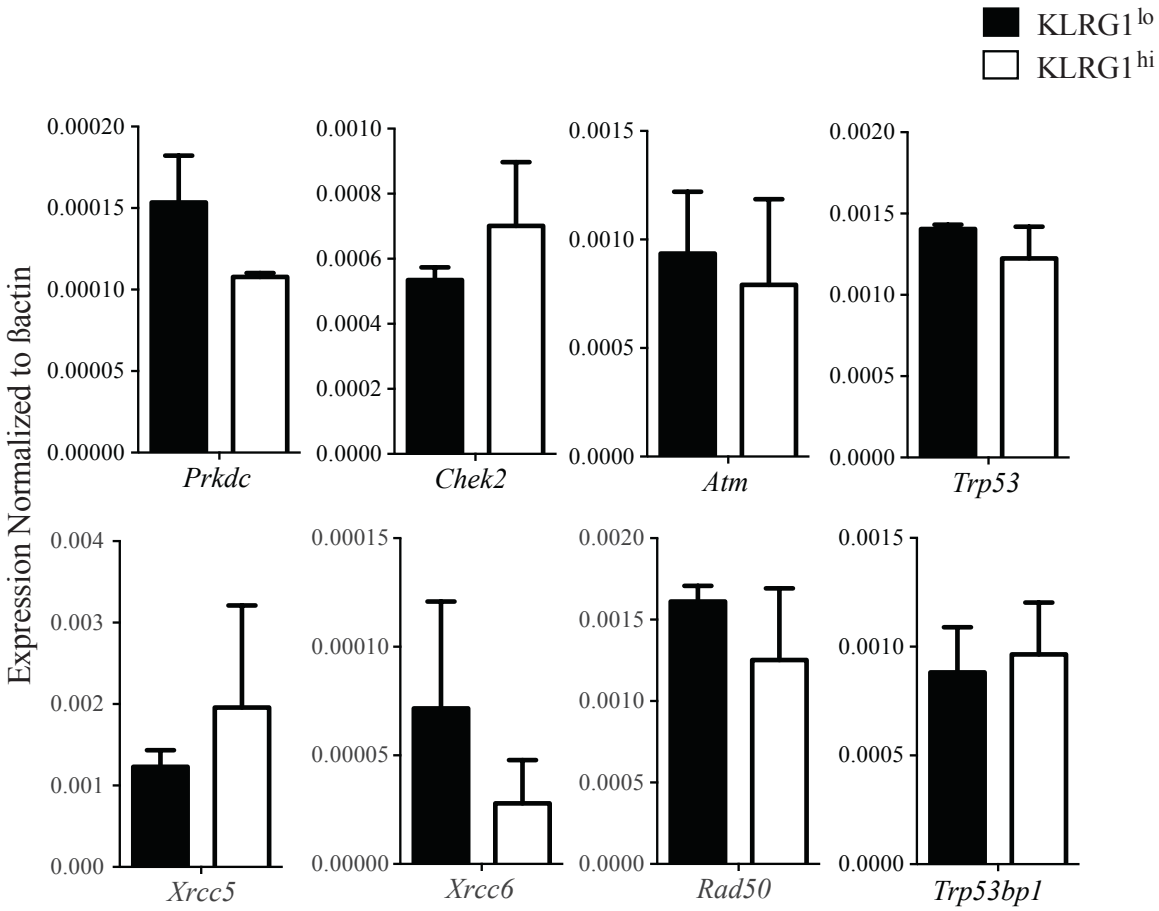

FIGURE 33 | DECREASED DDR TRANSCRIPT EXPRESSION IS NOT A HALLMARK TERMINAL DIFFERENTIATION

Transcript levels of various NHEJ factors were quantified by qRT-PCR in sorted KLRG1^{hi} (solid) and KLRG1^{low} (line) WT NK cells. Gene expression levels are normalized to β-actin. Results shown are representative of three independent experiments.

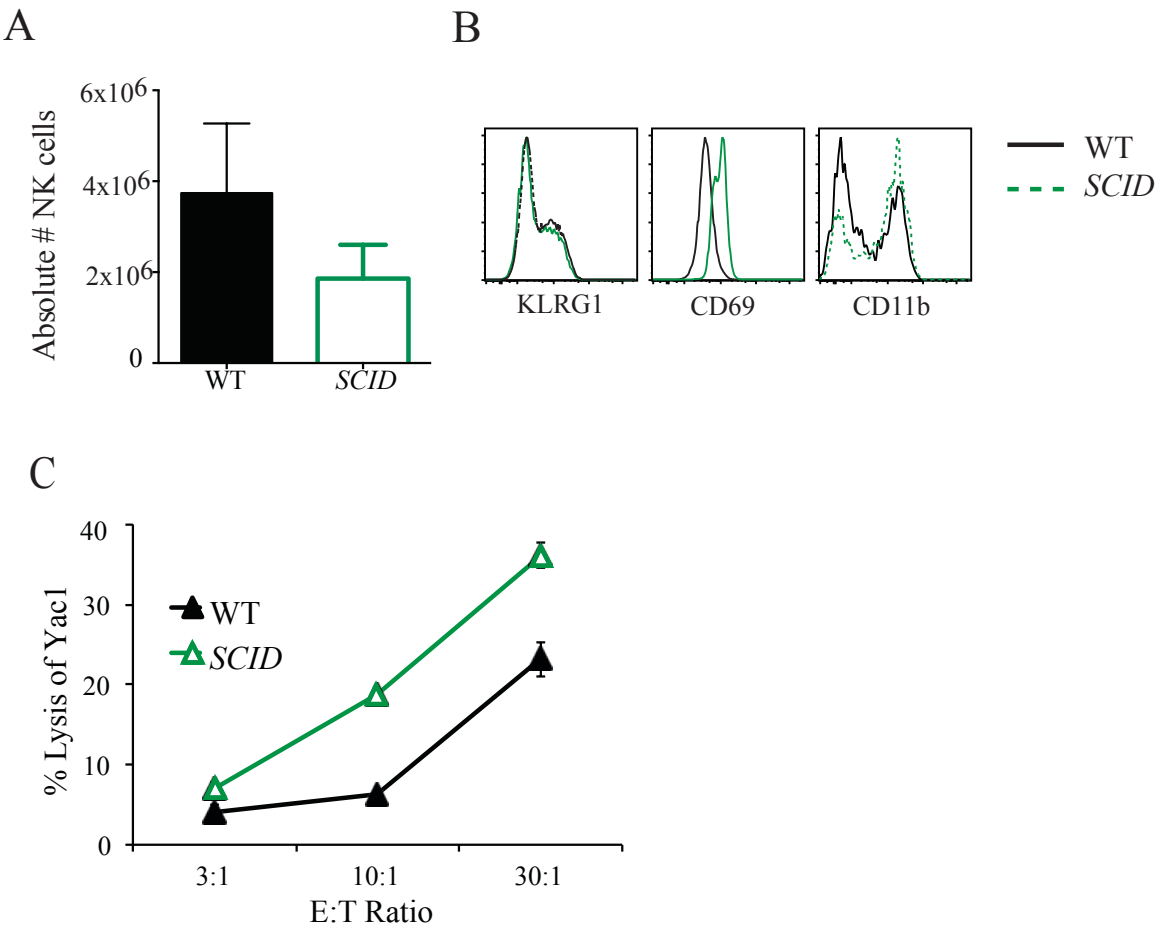

FIGURE 34 | *SCID* NK CELLS ARE HYPER-RESPONSIVE.

(A) Absolute numbers of splenic WT and SCID NK cells. Error bars show s.e.m. and data are representative of at least three independent experiments performed with n=3-5 mice. **(B)** Histograms show expression of activation markers on WT and SCID NK cells**. (C)** Percent lysis of ^{51}Cr-labeled Ba/F3-m157 (top) and Yac1 (bottom) target cells by WT and SCID NK cells *ex vivo*.

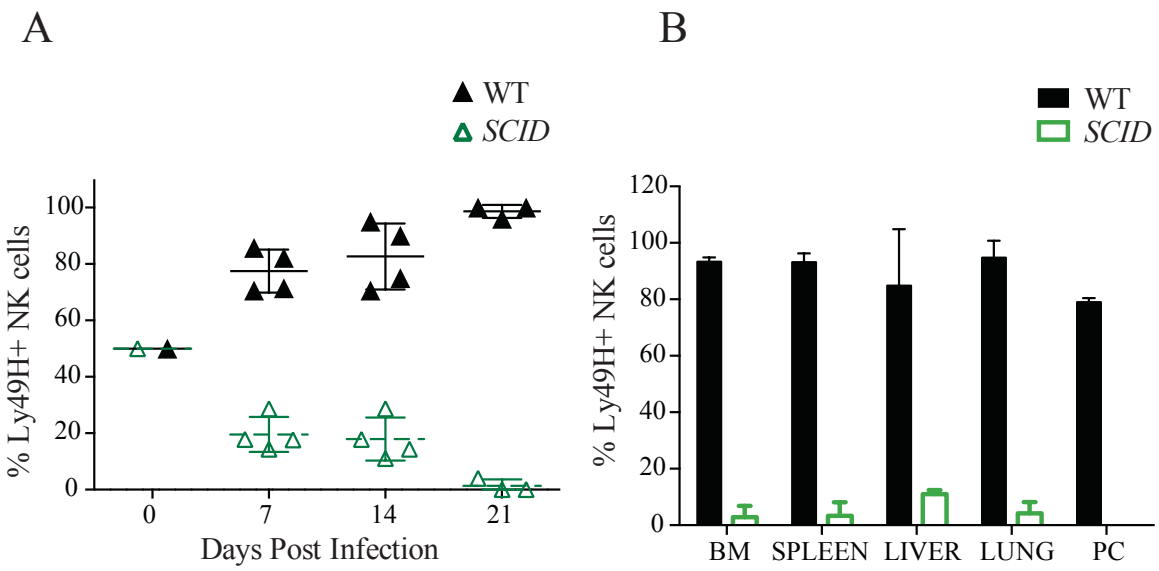

FIGURE 35 | DIMINISHED CELLULAR FITNESS *SCID* NK CELLS FOLLOWING MCMV INFECTION.

(A) Equal numbers of WT (CD45.1) and *SCID* (CD45.2) Ly49H^{+} NK cells were co-transferred into *Ly49h*$^{-/-}$ mice, and following MCMV infection the relative percentages of adoptively transferred populations in peripheral blood are shown for various time points. Results shown are representative of three independent experiments (n=3-5 mice), with each data point representing an individual mouse. **(B)** Graph shows percentage of transferred WT and *SCID* Ly49H^{+} NK cells in indicated organs at day 21 PI. Error bars show s.e.m. and data are representative of three independent experiments (n=3-4 mice).

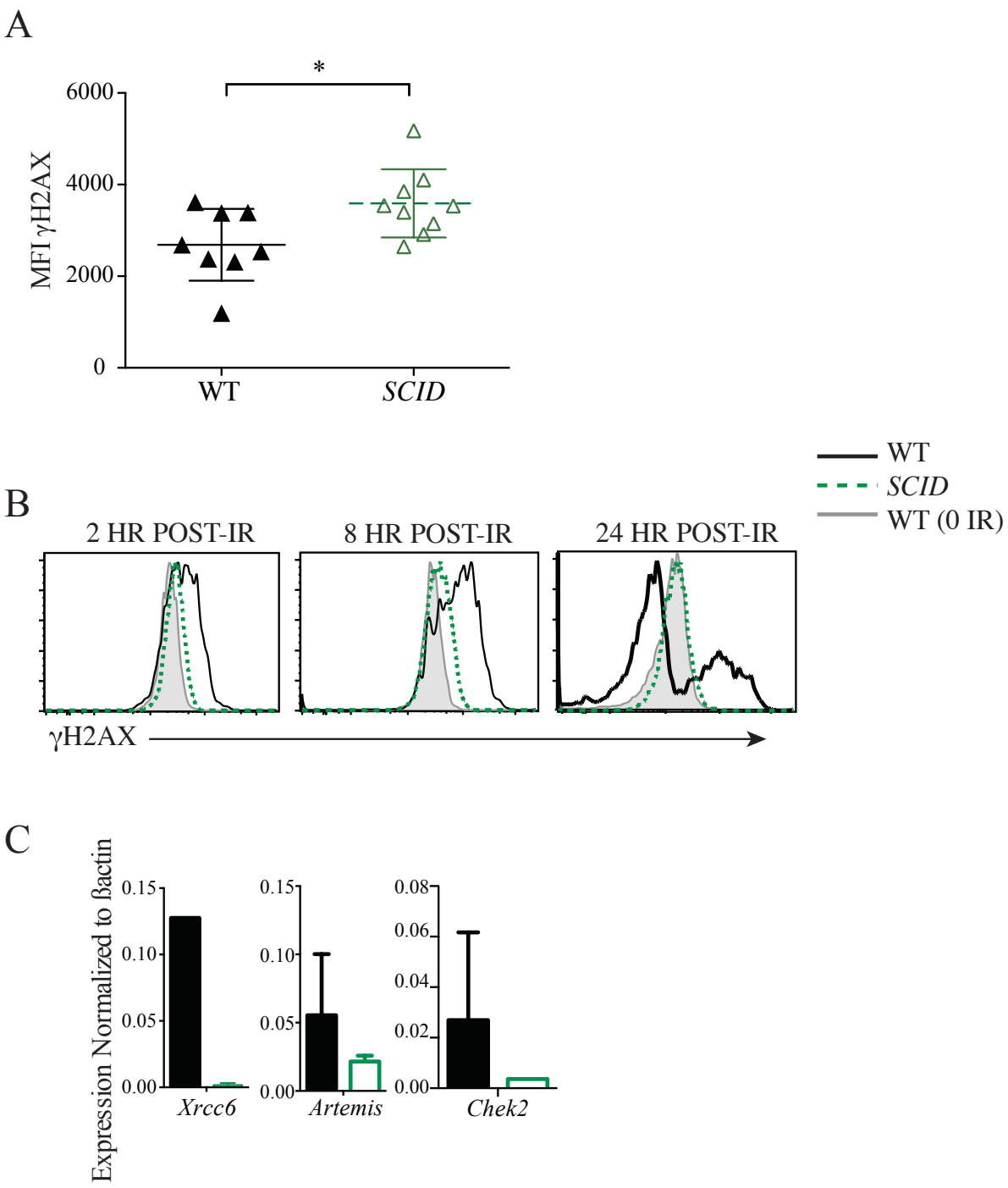

FIGURE 36 | *SCID* NK CELLS EXHIBIT REDUCED DNA BREAK REPAIR.

(A) MFI of γ-H2AX on splenic NK cells from WT and *SCID* mice. Data are representative of three independent experiments (n=5-8 mice). *$p < 0.05$. **(B)** WT and *SCID* NK cells from

mixed bone marrow chimeric mice were irradiated (IR, 10 Gy) and phosphorylation of H2AX was analyzed by flow cytometry at various time points post-IR. Representative histograms show irradiated WT (solid) and *SCID* (dashed) NK cells compared to unirradiated cells (0 IR, gray). **(C)** *Xrcc6, Artemis,* and *Chek2* mRNA levels were quantified by qRT-PCR in sorted WT and *Rag2*$^{-/-}$ NK cells. Gene expression levels are normalized to β-actin. Results shown are representative of three independent experiments.

We were able to further analyze other mice lacking essential elements of the NHEJ pathway: Ku80 (*Xrcc5*) and ATM. We found Ku80-deficient (*Xrcc5*$^{-/-}$) NK cells were more cytotoxic than their heterozygous (*Xrcc5*$^{+/-}$) littermates (Figure 37A*), recapitulating phenotypes we saw in *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ NK cells. Furthermore, adoptive transfer of ATM-deficient NK cells showed diminished survival following MCMV infection compared to WT NK cells (Figure 37B*). These data suggest that a fully functional DNA break/repair mechanism is essential for genomic stability of NK cells following virus-driven proliferation. Further experiments to determine the role of these NHEJ mediators and the DDR response in NK cells are underway.

Defective response of NK cells expressing catalytically-inactive RAG

To determine if the NK cell phenotype observed in RAG-deficient mice is due to the absence of RAG protein per se or the absence of RAG-mediated DNA cleavage, we analyzed active site RAG1 mutant mice on a *Rag1*-deficient background (*Rag1*$^{-/-}$ D708A) (Ji et al., 2010). The D708A mutant RAG1 protein expressed by these bacterial artificial chromosome transgenic mice lacks DNA cleavage activity but interacts with RAG2 and binds DNA normally (Fugmann et al., 2000a; Ji et al., 2010; Kim et al., 1999; Landree et al., 1999). Similar to *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ NK cells, NK cells from *Rag1*$^{-/-}$ D708A mice exhibited an activated and terminally differentiated phenotype at steady state and in mixed bone marrow chimeras (Figure 38A and data not shown). During MCMV infection, *Rag1*$^{-/-}$ D708A NK cells also exhibited impaired effector cell numbers

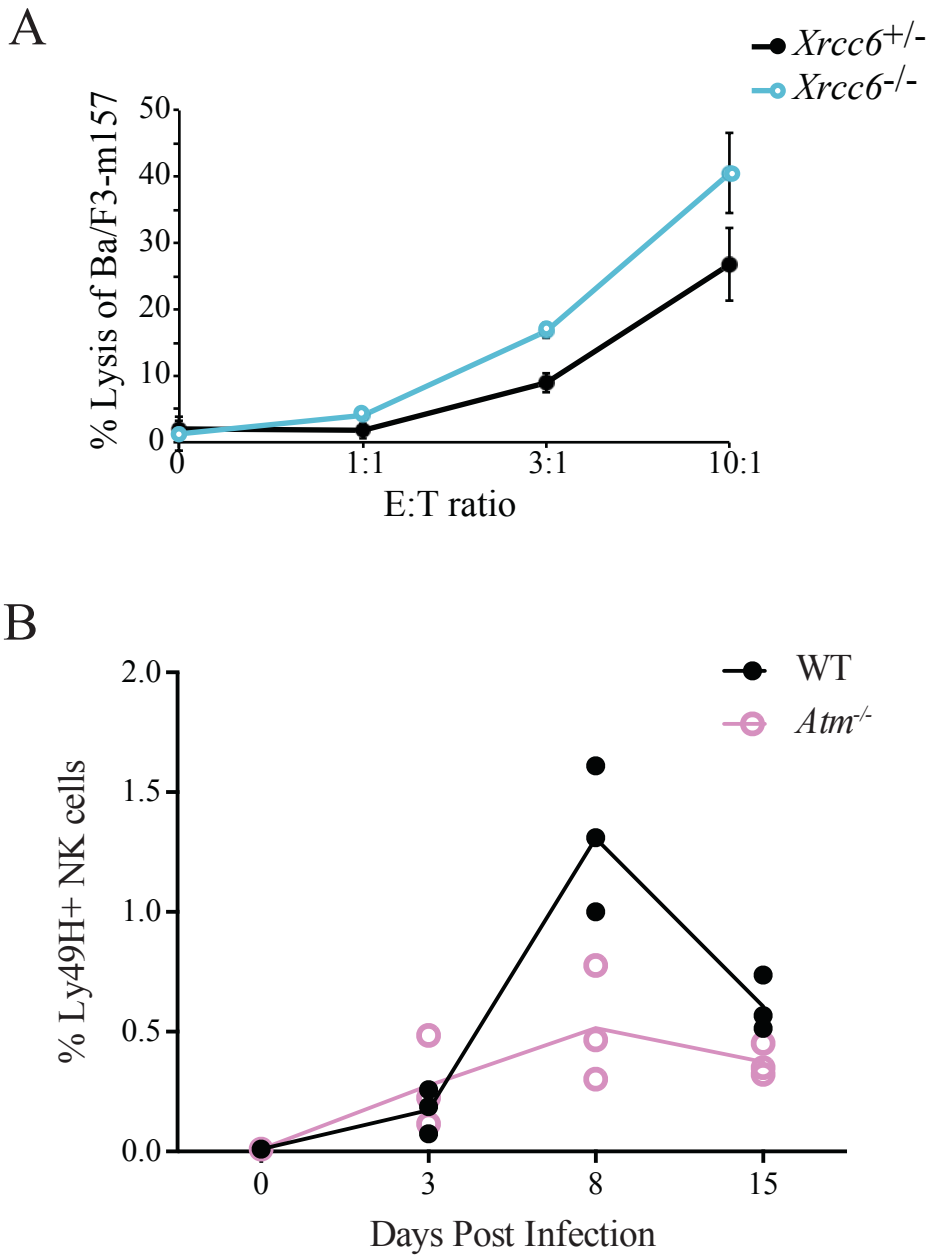

FIGURE 37 | NHEJ PROTEINS ARE NECESSARY FOR OPTIMAL NK CELL FUNCTION.

(A) Percent lysis of ^{51}Cr-labeled Ba/F3-m157 target cells by *Xrcc5*$^{+/-}$ and *Xrcc5*$^{+/+}$ NK cells *ex vivo*. **(B)** Equal numbers of WT (CD45.1) and *Atm*$^{-/-}$ (CD45.2) Ly49H^{+} NK cells were co-transferred into *Ly49h*$^{-/-}$ mice, and following MCMV infection the relative percentages of adoptively transferred populations in peripheral blood are shown for various time points. Results shown are representative of three independent experiments (n=3-5 mice), with each data point representing an individual mouse.

compared to WT NK cells (Figure 38B). Like *Rag1*$^{-/-}$ NK cells, *Rag1*$^{-/-}$ D708A NK cells contained higher levels of γ-H2AX at steady state compared to WT NK cells (Figure 38C). Thus, expression of catalytically-inactive RAG1 does not rescue the defective expansion, suggesting that RAG-mediated DNA cleavage during ontogeny is required for complete cellular fitness of NK cells responding to viral infection.

*Defective response of NK cells lacking RAG2 PHD Domain**

To further dissect the role of RAG2 during this process, we analyzed NK cells expressing a truncated RAG2 mutant (*Rag2*$^{c/c}$) (Deriano et al., 2011), lacking the H3K4me3 targeting PHD (Callebaut and Mornon, 1998; Ji et al., 2010; Matthews et al., 2007) domain and the phosphorylation site (T490) that targets RAG2 for destruction (Figure 3) (Li et al., 1996; Zhang et al., 2011). When crossed onto a p53-deficient background, T-lymphomas with translocations formed suggesting a role for the non-core RAG2 PHD domain in maintaining genomic stability (Deriano et al., 2011). Similar to *Rag2*$^{-/-}$ NK cells, NK cells from Rag2$^{c/c}$ mice exhibited an activated and terminally-differentiated phenotype at steady state and in bone marrow chimeras (Figure 39A). During MCMV infection, *Rag2*$^{c/c}$ NK cells also showed impaired effector cell numbers compared to WT NK cells (Figure 39B), however this phenotype was less dramatic showing it is not the only requirement for NK cell survival post MCMV infection. Like *Rag2*$^{-/-}$ NK cells, *Rag2*$^{c/c}$ NK cells showed lower transcript levels of *Prkdc* at steady state (Figure 39C). These results suggest RAG-mediated epigenetic modulation during ontogeny may be

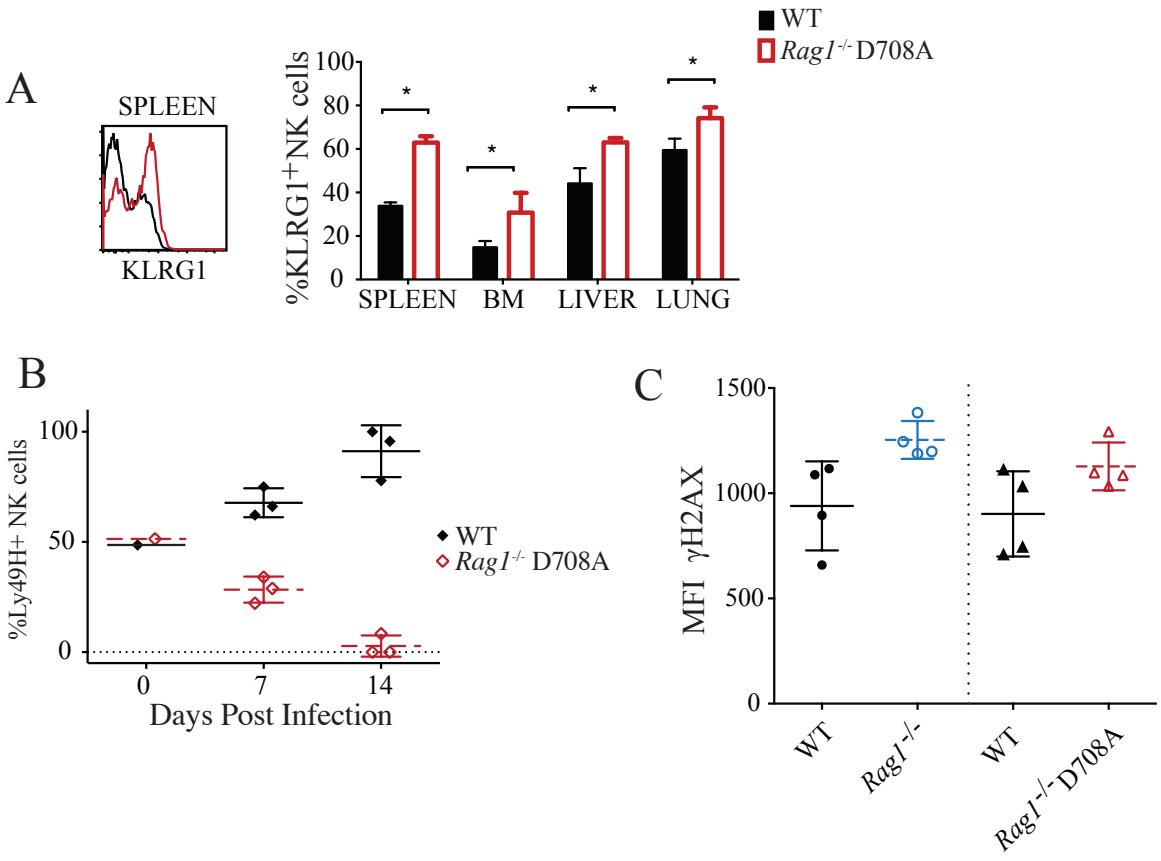

FIGURE 38 | DIMINISHED CELLULAR FITNESS IN HYPER-RESPONSIVE NK CELLS FROM *RAG1*$^{-/-}$ D708A MICE.

(A) NK cells from various organs of WT (CD45.1) and *Rag1*$^{-/-}$ D708A (CD45.2) bone marrow chimeric mice were analyzed for the activation and maturation markers shown. Error bars show s.e.m. and data are representative of three independent experiments (n=3-5 mice). *$p < 0.05$. **(B)** Ly49H^{+} NK cells from WT (CD45.1) and *Rag1*$^{-/-}$ D708A (CD45.2) mixed bone marrow chimeric mice were co-transferred into *Ly49h*$^{-/-}$ mice, and following MCMV infection the relative percentages of adoptively transferred populations in peripheral blood are shown for various time points. Results shown are representative of three independent experiments (n=3-5 mice). **(C)** MFI of γ-H2AX on splenic NK cells from WT:*Rag1*$^{-/-}$ (circles) and WT:*Rag1*$^{-/-}$ D708A (diamonds) bone marrow chimeric mice. Results are representative of three independent experiments (n=4-5 mice).

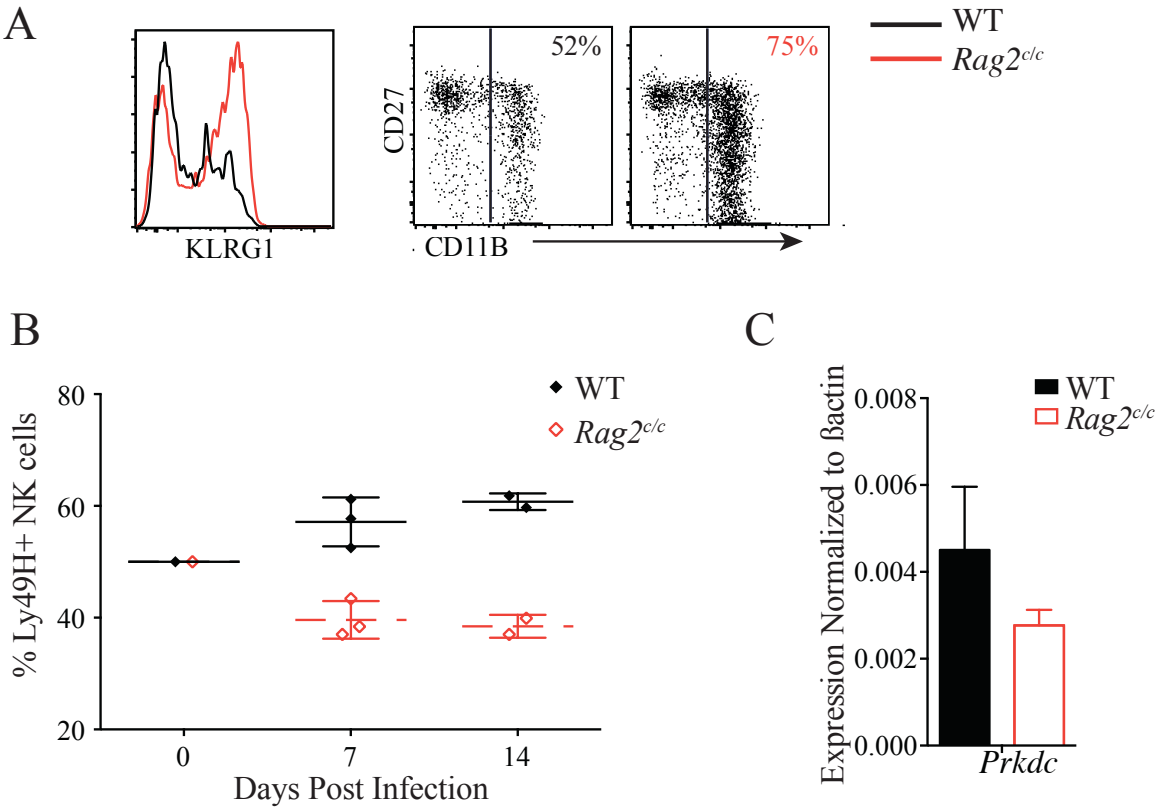

FIGURE 39 | NK CELLS LACKING THE RAG2 PHD DOMAIN HAVE A IMPAIRED RESPONSE TO MCMV INFECTION.

(A) NK1.1^{+} TCRβ^{-} NK cells from *Rag*$^{c/c}$ mice were analyzed for activation and maturation markers (KLRG1 and CD27/CD11b). Representative histograms and flow plots shown. **(B)** Equal numbers of WT (CD45.1) and *Rag2*$^{c/c}$ (CD45.2) Ly49H^{+} NK cells were co-transferred into *Ly49h*$^{-/-}$ mice, and following MCMV infection the relative percentages of adoptively transferred populations in peripheral blood are shown for various time points. (C) *Prkdx* mRNA levels were quantified by qRT-PCR in sorted WT and *Rag2*$^{c/c}$ NK cells. Gene expression levels are normalized to β-actin.

required for complete cellular fitness of NK cells responding to viral infection and expression of NHEJ proteins during development.

*RAG expression results in changes to epigenetic landscape in developing NK cells**

Using the RAG-YFG reporter, we saw no evidence that mature NK cells re-express RAG at any point during homeostasis or viral infection. Therefore, we believe RAG is acting during development to "imprint" cells through epigenetic or transcriptional changes. In RAG fate-mapping mice we observed that RFP+ NK cells had higher transcript level of *Chek2* (Figure 40A), compared to RFP- cells (similar to results seen in WT versus *Rag2*$^{-/-}$ mice). To further address whether the differential amounts of transcript were imprinted at the epigenetic level (i.e. more accessible promoter of *Chek2*), we performed H3K4me3 ChIP on RFP+ and RFP- NK cells sorted from our "fate mapping" system, and designed qPCR primers spanning a CNS region upstream of Chek2 (Figure 40B). H3K4me3, an activating histone mark typically found at accessible promoters of genes that are poised for transcription, showed enhanced expression in RFP+ NK cells compared to RFP- NK cells (Figure 40C). A negative control (gene desert) and positive control (*pGapdh*) were included. Altogether, these data suggest that RAG may act to "imprint" the NK cells with appropriate levels of DNA damage repair genes (such as *Chek2*), and that this may be regulated, at least in part, at the epigenetic level.

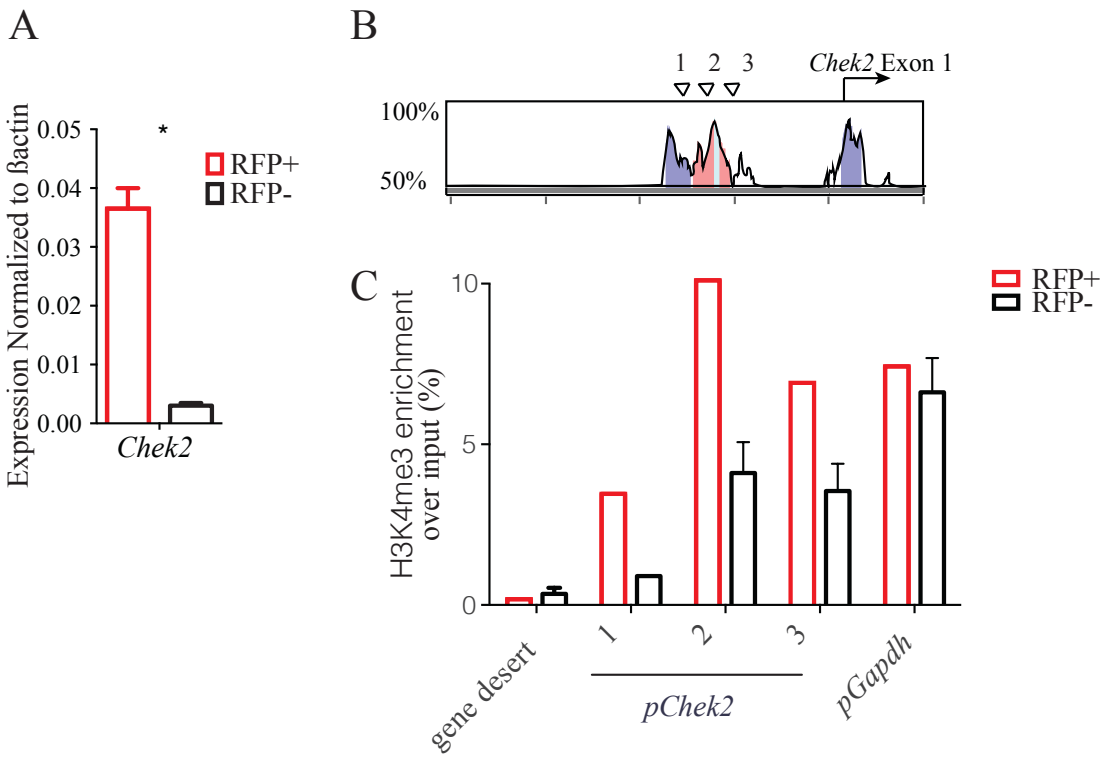

FIGURE 40 | EPIGENETIC DIFFERENCES EXIST BETWEEN NK CELLS WITH A HISTORY OF RAG EXPRESSION AND THOSE THAT DO NOT.

(A) *Chek2* mRNA levels were quantified by qRT-PCR in sorted WT and *Rag2*$^{c/c}$ NK cells. Gene expression levels are normalized to β-actin. **(B)** Vista browser image of CNSs (pink shading) in the mouse *Chek2* promoter. Primer sets used delineated by "1, 2, 3". **(C)** Enrichment of tri-methylated lysine 27 histone 3 (H3K4me3) at the *Chek2* promoter, as assessed by ChIP followed by qPCR on sorted WT NK cells (n = 3 biological replicates) from RAG "Fate-mapping" mouse. H3K4me3 occupancy on target (*Chek2* promoter (*pChek2*)) or control DNA (Gapdh promoter (*pGapdh*) or 'gene desert' ~50 kb upstream of Foxp3 gene) is expressed as percentage of input.

RAG expression in adaptive lymphocytes confers cellular fitness

To investigate the consequence of RAG-mediated genomic stability in other lymphocyte populations during ontogeny, we compared TCR transgenic $CD8^+$ T cells from OT-1 (RAG-competent) versus OT-1 x *Rag2*$^{-/-}$ mice (Hogquist et al., 1994). Although the OT-1 TCR is expressed early during thymocyte development in these mice, RAG proteins are still expressed in the RAG-competent transgenic background (using RAG^{GFP} reporter mice; P.J. Fink, personal communication); thus, the OT-1 system provides an opportunity to investigate T cell fitness in the presence or absence of RAG expression beyond its role in gene rearrangement at the antigen receptor loci. As with WT NK cells, $CD8^+$ T cells from OT-1 mice upregulated γ-H2AX within 2 hours of radiation treatment, followed by clearance of γ-H2AX foci within 24 hours (Figure 41A). In contrast, $CD8^+$ T cells from OTI-1 x *Rag2*$^{-/-}$ mice showed a delay in upregulation of γ-H2AX and a marked defect in their ability to clear γ-H2AX foci, indicating an inability to repair DSBs (Figure 41A, dashed line). Furthermore, similar to *Rag2*$^{-/-}$ NK cells, $CD8^+$ T cells from OT-1 x *Rag2*$^{-/-}$ mice possessed lower transcript levels of DNA-PKcs, Ku80, and ATM as compared to RAG-sufficient OT-1 $CD8^+$ T cells (Figure 41B). Together, these findings suggest that NK and T lymphocytes that lack RAG activity during ontogeny are predisposed to genomic instability due to decreased levels of essential DNA damage repair proteins.

To determine whether RAG expression confers fitness in T cells, we transferred equal numbers of OT-1 and OT-1 x *Rag2*$^{-/-}$ $CD8^+$ T cells into B6 mice and determined survival

following infection with a recombinant MCMV expressing Ova. We found that OT-1 x *Rag2*$^{-/-}$ cells possessed a reduced capacity for survival compared to OT-1 cells (Figure 41C), similar to our observations in NK cells. Furthermore, *in vitro* cultured OT-1 and OT-1 x *Rag2*$^{-/-}$ CD8^{+} T cells stimulated with Ova peptide (SIINFEKL) or anti-CD3/CD28 beads yielded a similar result (Figure 41D-E), demonstrating that RAG expression during ontogeny endows peripheral T cells with enhanced cellular fitness.

RAG expression in ILCs confers cellular fitness

Innate lymphoid cells (ILC) have recently been described to maintain tissue homeostasis and provide protection against pathogens at mucosal surfaces (e.g. gut, lung, adipose tissue). (Artis and Spits, 2015; Cortez et al., 2015). Similar to T, B, and NK cells, ILCs develop from the CLP and require the common gamma chain for their survival. ILCs have been divided into three classes based on their transcription factor requirements, response to cytokines, cytokine production, and anatomical location. Group 1 ILCs (ILC1) produce IFN-γ in response to IL-12 and IL-18, and are broadly found throughout lymphoid and non-lymphoid organs (e.g. liver, intestine, spleen, peritoneal cavity). ILC1 can be distinguished from NK cells by transcription factors requirements of T-bet but not Eomes, and are important for immunity against viruses, intracellular bacteria and parasites. Unlike NK cells, ILC1 are not thought to mediate cytotoxicity. Group 2 ILCs (ILC2) require GATA3, RORα, and GFI1 expression, produce type 2 cytokines (IL-4, IL-5, and IL-13) and amphiregulin in response to IL-25 and IL-33, and are predominantly found in the lung and adipose tissue to protect against helminth infection. Group 3 ILCs

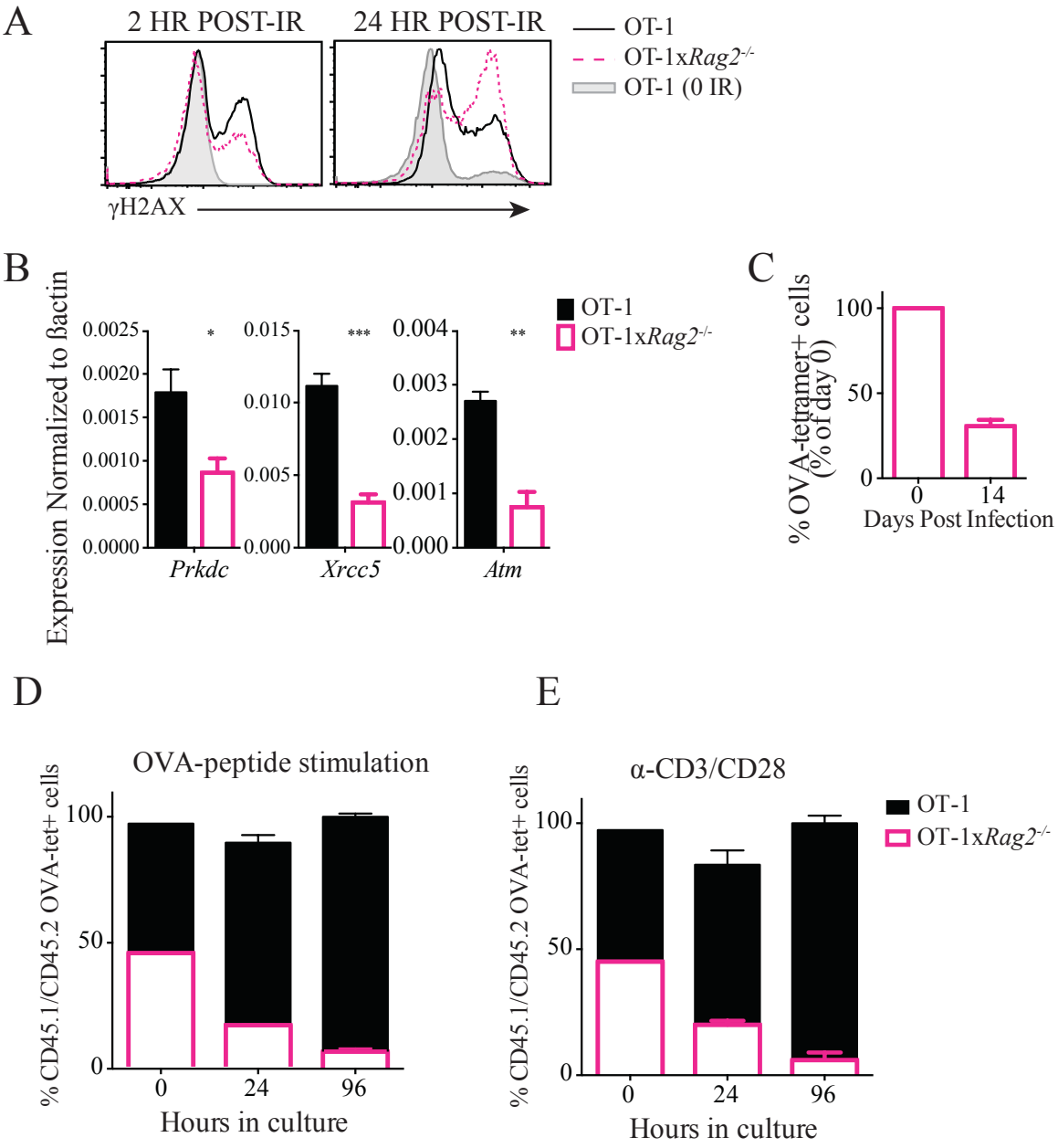

FIGURE 41 | A NOVEL ROLE FOR RAG IN THE CELLULAR FITNESS OF CD8+ T CELLS.

(A) $CD8^+$ T cells from OT-1 and OT-1 x *Rag2*$^{-/-}$ mice were irradiated (10 Gy) and phosphorylation of H2AX was analyzed by flow cytometry at various time points post-IR. Representative histograms show irradiated OT-1 (solid) and OT-1 x *Rag2*$^{-/-}$ (dashed) $CD8^+$ T cells compared to unirradiated cells (0 IR, gray). **(B)** *Prkdc, Xrcc5,* and *Atm* mRNA levels were quantified by qRT-PCR in sorted $CD8^+$ T cells from OT-1 and OT-1 x *Rag2*$^{-/-}$ mice.

Gene expression levels are normalized to β-actin. Results shown are representative of three independent experiments. **(C)** Equal numbers of $CD8^+$ T cells from OT-1 (CD45.1x2) and OT-1 x *Rag2*$^{-/-}$ (CD45.1) mice were adoptively co-transferred into B6 mice (CD45.2) and relative percentage of OT-1 x *Rag2*$^{-/-}$ cells to OT-1 cells were determined following MCMV-Ova infection. **(D-E)** Equal numbers of OT-1 (CD45.1) and OT-1 x *Rag2*$^{-/-}$ (CD45.2) T cells were co-cultured for 4 days in the presence of 1 μM SIINFEKL peptide (D) or anti-CD3/CD28 beads (E). Graphs show ratio of $CD45.1^+$ and $CD45.2^+$ T cells, determined at indicated time points by flow cytometry.

(ILC3) require RORγt and T-bet, and are important for immunity against extracellular bacteria. ILC3 are found primarily in the gut, and produce a variety of cytokines, including IL-17 and IL-22, in response to IL-1β and IL-23 (Artis and Spits, 2015). Although the common gamma chain cytokine receptor and cytokine IL-7 are required for ILC development, it is thought RAG proteins are not essential (Spits and Cupedo, 2012).

We examined group 2 and 3 ILCs in our RAG fate-mapping mice and observed that 30-50% of these cells had a history of RAG expression during ontogeny (Figure 42A-B), consistent with a previous report on ILC2s (Yang et al., 2011). Analysis of the NK lineage in RAG2^{YFP} knockin reporter mice (Igarashi et al., 2001; Kuwata et al., 1999) revealed RAG expression in ILC-precursor (FLT3-α4β7+) in bone marrow, but not in mature ILC2 or 3 in the periphery (Figure 42C-D). In addition, ILCs from *Rag2*$^{-/-}$ mice possessed higher γ-H2AX levels at steady state compared with WT mice (Figure 43). Whether there is a functional consequence of the RAG-based heterogeneity in ILC populations at mucosal barriers is currently being investigated.

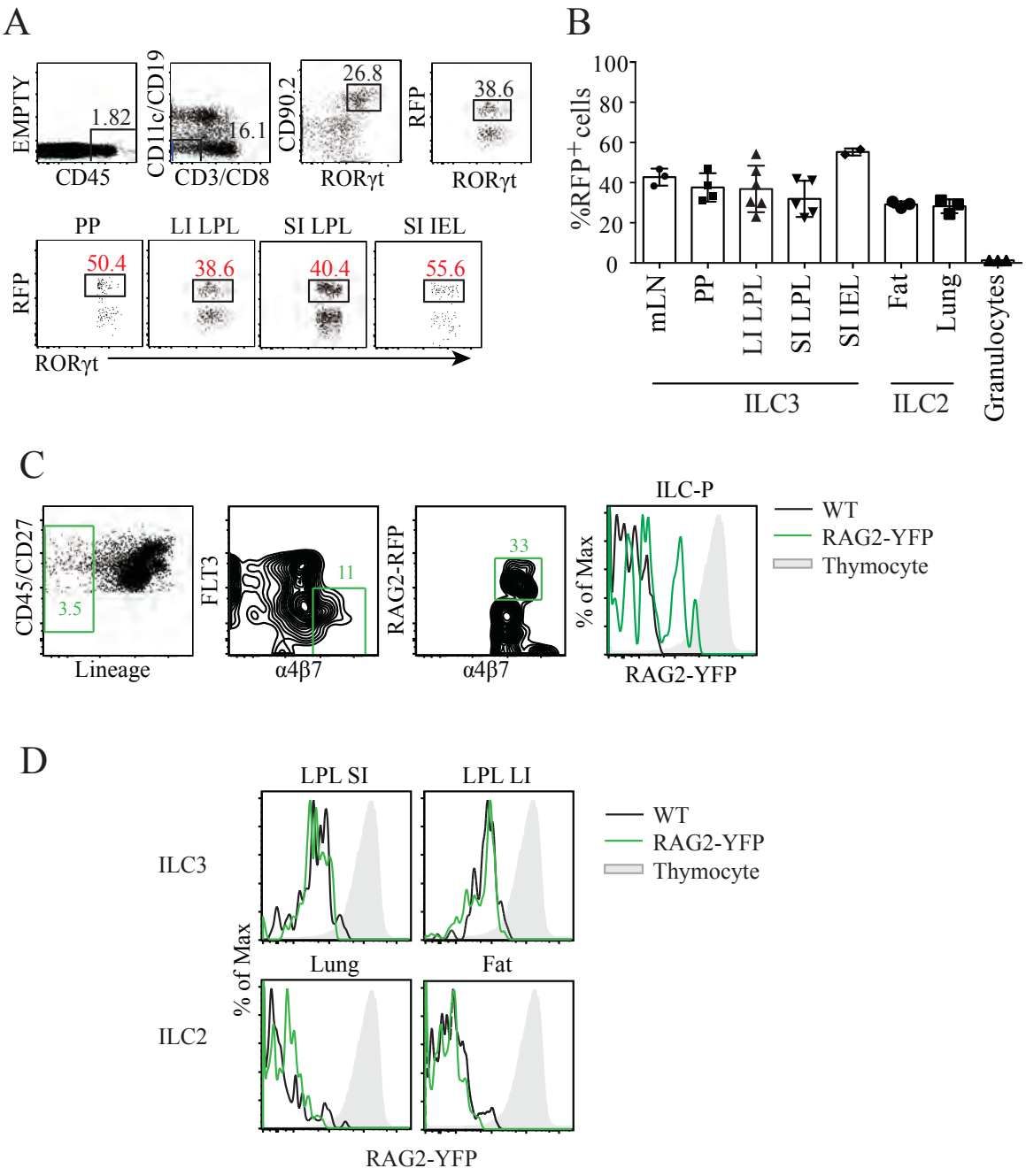

FIGURE 42 | A NOVEL ROLE FOR RAG IN THE CELLULAR FITNESS OF INNATE LYMPHOID CELLS.

(A-B) Group 2 and 3 innate lymphoid cells **(**ILC2 and ILC3) were isolated from various organs of RAG fate-mapping mice, and percentage of RFP^+ cells was determined by flow cytometry. Gating strategy for intestinal ILC3 (A) and quantified percentages of ILC3 and

ILC2 in the indicated tissues are shown (B). **(C-D)** Flow cytometric analysis of GFP expression was performed on bone marrow of *Rag2*^{GFP} mice to identify ILC-Precursor (FLT3^{-} α4β7^{+}) within the Lin^{-} CD27^{+} CD45^{+} cell population (C); and intestinal mature ILC3 using gating strategy shown in (A) and CD90^{+}GATA3^{+} to identify ILC2 within Lin^{-}CD45^{+} population (D). Lin^{-} (or Lineage-negative) is defined as CD19^{-} CD3^{-} TCRβ^{-} CD4^{-} CD8^{-} Ter119^{-}.

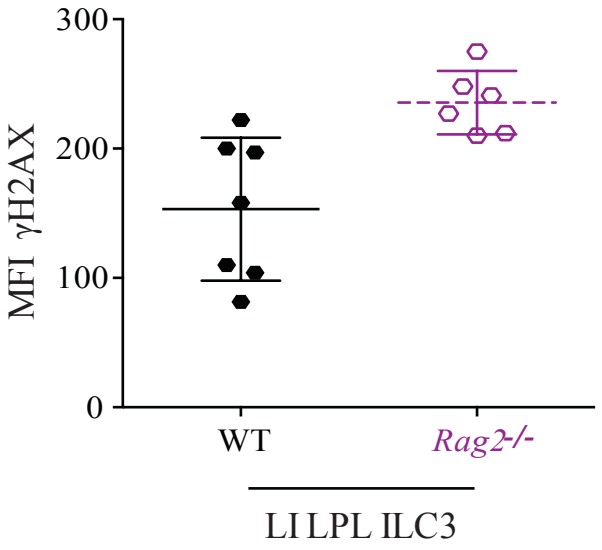

FIGURE 43 | NK CELLS THAT LACK RAG EXPERISSION HAVE HIGHER GENOMIC INSTABILITY.

Mean fluorescence intensity (MFI) of γ-H2AX staining of intestinal ILC3s from WT and *Rag2*$^{-/-}$ mice. Data are representative of three independent experiments (n=5-10 mice), with each data point representing an individual mouse.

*RAG-expressing ILCs preferentially expand during C. Rodentium infection**

ILC3s have been shown to be critical for host protection against the murine enteric pathogen *Citrobacter rodentium*, as mice lacking ILC3s or depleted of ILCs become susceptible to bacterial dissemination and mortality (Sonnenberg et al., 2012; Sonnenberg et al., 2011). To examine a functional consequence of RAG expression in ILCs, we examined whether the percentage of RFP-expressing ILC3s would modulate following *C. rodentium* challenge in RAG fate-mapping mice (Figure 44A). At d6 post infection, we saw an increase in RFP+ ILC3 in the small intestine (SI) and large intestine (LI) compared to uninfected (Figure 44B). The higher percentage of RFP+ ILC3s in the LI may correlate with *C. rodentium* being found in the cecum, and suggests ILCs that have a history of RAG expression have higher 'fitness' during *C. rodentium* infection. Previous studies have shown ILCs do not undergo extensive proliferation during *C. rodentium* infection (Geiger et al., 2014). Therefore whether these cells undergo site-specific proliferation or are preferentially recruited to the site of infection remains to be determined. Further studies to understand the role of RAG in ILCs, including cytokine production and response to other pathogens and infections, are underway.

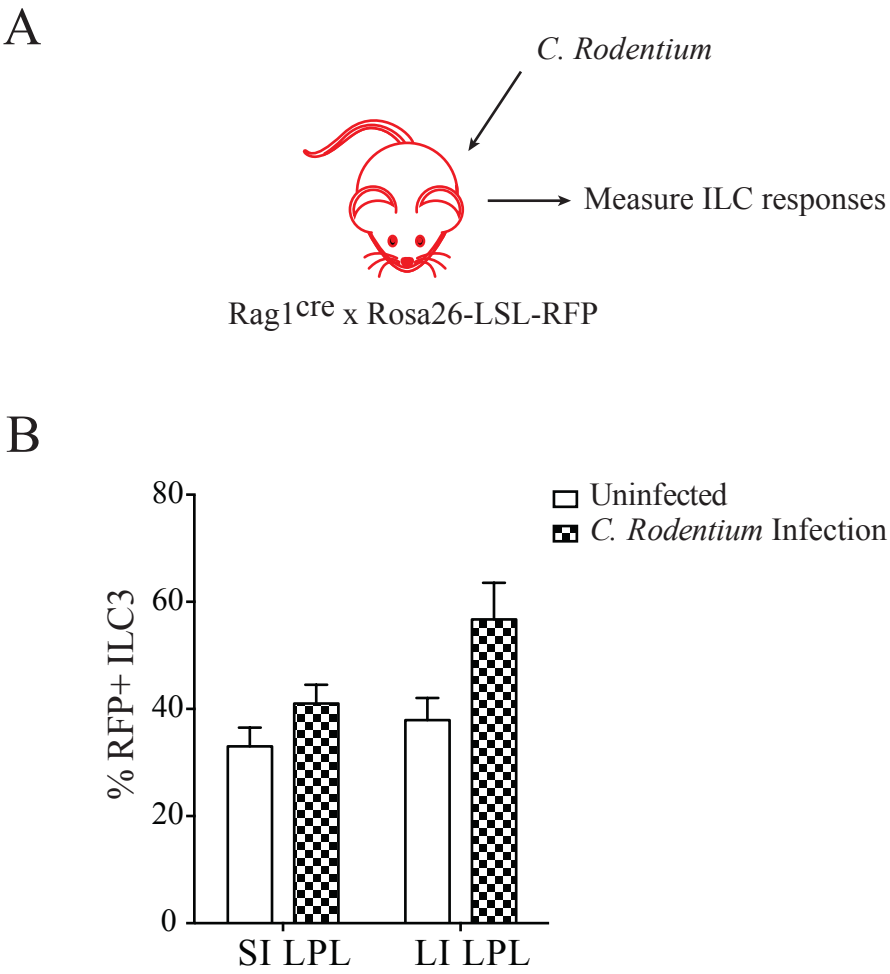

FIGURE 44 | ILCS WITH A HISTORY OF RAG EXPRESSION EXPAND DURING *C. RODENTIUM* INFECTION.

(A) Schematic of *C. rodentium* experiment. **(B)** %RFP in uninfected and infected ILC3 populations of RAG 'fate-mapping' mice. Mice were analyzed at d6 post *C. Rodentium* infection. All data are representative of n = 3-5 mice per group, with error bars showing SEM from independently repeated experiments.

Discussion

Although the mechanism behind stochastic expression of RAG in developing lymphocytes (only half in bone marrow show evidence of previously expressing RAG) is not known, mutant NK cells lacking RAG (or RAG activity) or WT NK cells lacking a history of RAG expression are characterized by greater apoptosis and γ-H2AX staining at steady state, and an inability to remove the γ-H2AX foci following DNA damage. In contrast, WT NK cells with a history of RAG expression a can generate long-lived memory cells following antigen-specific proliferation, characterized by increased survival and ability to repair DNA DSBs. These differences are further stratified by observed differences in DDR proteins, where RAG-deficient NK cells see significantly lower levels of essential NHEJ proteins. Hence, there exists an unexpected functional dichotomy between NK cell populations, in which RAG expression and likely RAG-generated DNA breaks are used for the purpose of creating a long-lived NK cell pool to combat pathogens.

While we focus on programmed DSB and deficient repair in RAG-deficient NK cells as the root cause of increased γH2AX levels at steady state, many other processes cause increased H2AX foci formation. For example, reactive oxygen species (ROS) are ions or small molecules produced by normal metabolic processes and exogenous agents that cause DNA damage (Tanaka et al., 2007; Vilenchik and Knudson, 2003). Accumulation of unrepaired DNA damage induced by ROS is implicated in cell aging and neurodegenerative disease. Further, DNA damage from various sources has been shown

to increase ROS levels (Rowe et al., 2008). The exact mechanism by which DNA damage induces ROS and the involvement of DDR proteins in ROS generation remains to be elucidated, however one could hypothesize that if increased DNA damage in the RAG-deficient NK cells resulted in increased levels of ROS, this may contribute to the diminished fitness and survival of these cells. In future studies, we will investigate steady state ROS levels in RAG-deficient and WT NK cells to determine if there is a link between ROS expression, γH2AX foci formation, and the decreased cellular fitness observed in lymphocytes that have not expressed RAG.

Consistent with the finding that poorly surviving RAG-deficient NK cells possess diminished levels of DNA-PK, NK cells from *SCID* mice (possessing a genetic mutation in DNA-PK) also have an expansion defect following viral infection. Interestingly, DNA-PK-deficient mouse embryonic fibroblasts (MEFs) display a higher frequency of spontaneous chromosomal aberrations compared to WT at steady state and this difference increases upon irradiation (Ferguson et al., 2000; Martin et al., 2005). In humans, reduced DNA-PK activity has been associated with lung cancer (Auckley et al., 2001), uterine, cervix, and breast cancer (Someya et al., 2006). DNA-PK-mediated genomic instability is also due to impaired telomere maintenance as DNA-PKcs-deficient mice display high levels of telomere fusions (Gilley et al., 2001). Telomeres are found on the ends of chromosomes as protective caps to prevent fusion to other chromosomes or deterioration (Blackburn, 2000). Terminal differentiation of specialized cell types (as seen in RAG-deficient NK cells) also results in a loss in telomerase activity (Weissman, 2000). Therefore, lack of DNA-PK expression in RAG-deficient NK cells further adds to the

genomic instability seen in these cells. Deeper analysis of chromosomal aberrations within NK cells and the role of DNA-PK remain to be determined.

Moreover, the promoter of DNA-PK contains the promoter region of mini-chromosome maintenance-deficient 4 (MCM4), a protein that is divergently transcribed (Connelly et al., 1998). MCM4 encodes a highly conserved mini chromosome maintenance helicase that is essential for DNA replication (Forsburg, 2004) and is important for maintaining genome stability (Shechter and Gautier, 2004). Interestingly, cohorts of Irish patients were found to have a mutation in *Mcm4* resulting in an NK cell deficiency (Gineau et al., 2012; Hughes et al., 2012). These patients were found to have an overabundance of immature NK cells in bone marrow (Hughes et al., 2012); however, this phenotype may be due to a defect in survival of the terminally differentiated NK cells (due to decreased genome stability associated with MCM4 deficiency), rather than a defect in development. Further, these patients were not analyzed for defects in DNA-PK *(Prkdc)*, which may have resulted from *Mcm4* mutations. These studies further highlight the importance for genomic stability maintenance in NK cells.

Although our findings suggest that selective RAG expression in developing lymphocytes confers resistance to DNA damage, the molecular mechanisms behind this preservation of cellular fitness at steady state and during cellular stress remain unclear. Because the *Rag1*$^{-/-}$ D708A NK cells (possessing a RAG1 catalytic mutant (Ji et al., 2010)) showed a similar defect to *Rag1*$^{-/-}$ NK cells during MCMV infection, we infer that RAG-mediated cleavage events during NK cell ontogeny are essential for initiating the induction of DNA

repair enzymes, thereby "educating" the developing NK cell to better respond to subsequent DNA damage as a peripheral mature lymphocyte. The prediction that a "feed forward" mechanism exists in developing lymphocytes, whereby DNA DSBs and recruitment of DNA damage response proteins results in a downstream enhancement of DDR later in life, remains to be further tested.

RAG2 is known to possess highly conserved domains that are important for maintaining genome integrity (Fugmann et al., 2000a). Specifically, the RAG2 C-terminus domain is required to escort the post-cleavage RAG:DNA complex into the canonical NHEJ pathway for proper DNA repair (Fugmann et al., 2000a; Lee et al., 2004). Genetic ablation of only the "non-core" domain in *Rag2*$^{c/c}$ mice (Liang et al., 2002) (which still contains functional cleavage activity) has been shown to induce genome-wide chromosomal aberrations and lymphomagenesis when crossed to highly proliferative *p53*$^{-/-}$ mice (Deriano et al., 2011). *Rag2*$^{c/c}$ cells show a similar, albeit reduced, survival defect to *Rag2*$^{-/-}$ NK cells during MCMV infection, suggesting the PHD domain of RAG2 plays a role in the "fitness" of NK cells. Because this phenotype is not as pronounced as *Rag2*$^{-/-}$ or *Rag1*$^{-/-}$ D708A NK cells, this suggests the PHD domain is not as essential as the catalytic functions of RAG in determining cellular fitness. Whether additional "non-core" functions of RAG activity (beyond the catalytic cleavage of DNA) further endow innate lymphocytes, such as NK cells, with greater genomic integrity remain to be elucidated.

A close correlation has also been reported between RAG2 binding and the activating H3K4me3 histone mark at the promoters of many tissue specific genes throughout the

genome (Ji et al., 2010; Liu et al., 2007; Matthews et al., 2007). Therefore, epigenetic analysis of regulatory regions surrounding DNA damage repair genes in WT compared to RAG-deficient NK cells may provide mechanistic insights into how damage response protein levels are set in an individual resting lymphocyte. In conjunction with this, we observed a difference in H3K4me3 modification at the *Chek2* promoter, a kinase downstream of ATM essential for DDR (Reinhardt and Yaffe, 2009), in NK cells with or without a history of RAG expression. In RFP- NK cells from the RAG fate mapping mouse, the *Chek2* promoter showed lower levels of H3K4me3, suggesting this promoter is less poised for transcription or in a "closed" confirmation, compared to RFP+ cells that had expressed RAG. Therefore, it is possible that the RAG1/2 complex plays a regulatory function during ontogeny at sites lacking RSS sequences (e.g. DDR proteins), resulting in changes in transcriptional activity, histone modifications, or chromatin structure of non-antigen receptor loci. Investigation of specific DDR promoters, as well as global changes in methylation, will begin to paint a more complete picture of the role of RAG in modulating the NK cell genome beyond its canonical recombination function.

Although we have demonstrated that RAG-deficiency results in a reduced cellular fitness in NK cells that is revealed during the response against viral infection, this novel role for RAG is not restricted to NK cells. The decrease in DNA-PKcs, Ku80, and ATM transcripts, and the aberrant upregulation and clearance of the γ-H2AX mark observed in NK cells from *Rag2*$^{-/-}$ mice, were also observed in OT-1 CD8^{+} T cells on a *Rag2*$^{-/-}$ background compared to WT OT-1 cells. Although *Rag2*$^{-/-}$ OT-1 cells did not persist as well as WT OT-1 cells following viral challenge, this survival defect was not as

pronounced as with *Rag2*$^{-/-}$ versus WT NK cells, suggesting that T cells possess additional mechanisms conferring longevity relative to NK cells. These data, along with our observation of differential RAG expression and γ-H2AX levels within ILC subsets, raise the possibility that RAG activity modulates cellular fitness in B cells, as well as in other "innate" lymphocyte populations (e.g. NKT cells, gd T cells, B1 B cells, and even DCs of lymphoid origin).

DNA damage is generally assumed to be a detrimental event, frequently associated with impaired cell survival or cellular transformation (Bednarski and Sleckman, 2012; Sherman et al., 2011; Zhang et al., 2011). However, a growing body of evidence suggests that focal DNA breaks may be a mechanism for normal cell development, differentiation, and function (further discussed in Chapter 5). Additional studies will investigate how stochastic RAG expression induces the DNA damage response proteins in lymphocytes during ontogeny and determines both the "set point" for DNA DSB repair enzymes in resting lymphocytes and the ability of activated and proliferating lymphocytes to repair DSBs. Irrespective of the precise molecular mechanism behind the RAG-endowed cellular fitness of lymphocytes, it is fascinating to envision a paradigm where a programmed DNA endonuclease like RAG is able to stably "imprint" the functional properties of a developmental lineage long after it has acted on it. Our findings identify a novel role for the RAG proteins and add to a growing body of evidence implicating DNA damage in the regulation of gene expression, cell development, and cell fate in eukaryotes (Abramson et al., 2010; Bredemeyer et al., 2008; Ju et al., 2006; Larsen and Megeney, 2010; Larsen et al., 2010a; Schroeder et al., 2013).

CHAPTER 5: DISCUSSION

The appearance of the RAG in jawed vertebrates during evolution endowed T and B cells with the ability to mediate V(D)J gene rearrangement at their antigen receptor loci, providing these lymphocytes with a molecular mechanism for diversifying their antigen receptor repertoire. In contrast to these adaptive immune cells, NK cells classically represent the third lineage of lymphocytes (i.e. innate lymphocytes) that possess germ-line-encoded antigen receptors and do not require RAG for their development (Kondo et al., 1997). Thus, since their discovery 40 years ago, NK cells are placed in immunology textbook chapters devoted to the innate immune system (Murphy, 2012).

However, this classical view of NK cells has been rapidly changing in last decade. Recent evidence suggests that this cell type possesses traits attributable to adaptive immunity (Sun and Lanier, 2011; Vivier et al., 2011). These characteristics include education mechanisms to ensure self-tolerance during NK cell development (Orr and Lanier, 2010), and clonal-like expansion of antigen-specific NK cells during viral infection followed by the ability to generate long-lived progeny known as “memory” NK cells (Daniels et al., 2001; Dokun et al., 2001; Sun et al., 2009, 2010). NK cell memory has also been described in a plethora of non-pathogen settings (Cooper and Yokoyama, 2010; Paust and von Andrian, 2011). In addition, NK cells share many specific similarities with CD8+ T cells, including their development from the common lymphoid progenitor (CLP), their

requirement for the IL-2 common gamma chain for their survival, their expression of similar activation/maturation markers, and their use of identical cytolytic machinery (perforin and granzymes) to destroy transformed or virally-infected target cells (Sun and Lanier, 2011). Some of the underlying molecular mechanisms that control NK cell function and longevity, resulting in effector and memory NK cells subsets during pathogen challenge, have only recently come to light.

This thesis demonstrates that RAG expression during adaptive and innate lymphocyte development results in functional heterogeneity in the periphery. Those NK cells that have a history of RAG expression are able to mount a successful memory response to MCMV due to enhanced DNA damage repair, suggesting a role for the RAG recombinase and the DDR beyond their canonical pathways. Irrespective of the precise molecular mechanism behind the RAG-endowed cellular fitness of lymphocytes, these examples suggest a paradigm where controlled DNA damage is able to alter the functional properties of a developmental lineage during ontogeny, leading to alterations that condition subsequent cellular behavior.

RAG expression induces functional heterogeneity

Using the RAG fate mapping mice, NK cells that never expressed RAG during development (RFP^-) were more terminally differentiated (as defined by greater KLRG1 expression), and demonstrated a higher degree of cytotoxicity (Karo et al., 2014). We

investigated whether RAG influenced *in vivo* NK cell responses by incorporating a well-established viral model of antigen-specific NK cell expansion (Sun et al., 2009). NK cells expressing the mouse cytomegalovirus (MCMV)-specific activating receptor Ly49H will undergo a robust antigen-driven proliferation (100-1000 fold expansion) following MCMV infection, and following viral clearance, a population of long-lived memory NK cells persists in both lymphoid and non-lymphoid organs (Sun et al., 2009). Donor NK cells from RAG1 or RAG2-deficient mice were outcompeted by WT NK cells following adoptive transfer and MCMV infection. NK cells that lacked either RAG or a history of RAG expression were defective in virus-driven expansion and failed to persist due to an increase in apoptosis (Karo et al., 2014) (Figure 45). These results suggest a role for RAG outside of V(D)J recombination in NK cell development that results in enhanced cellular "fitness" seen in peripheral NK cells.

How RAG expression during NK cell ontogeny established functional heterogeneity in mature NK cells remained to be determined. We observed NK cells that lacked RAG expression have a decreased expression in essential components of the DDR including DNA-PKcs (*Prkdc*), Ku80 (*Xrcc5*), Chk2 (*Chek2*), and ATM (*Atm*) in mature cells. These perturbations in gene expression strongly correlated with inefficient DNA repair, as measured by γ-H2AX foci appearance and removal, following introduction of exogenous DNA damaging agents such as radiation. Interestingly, this phenomenon was not restricted to innate lymphocytes. Using TCR transgenic $CD8^+$ T cells from OT-1

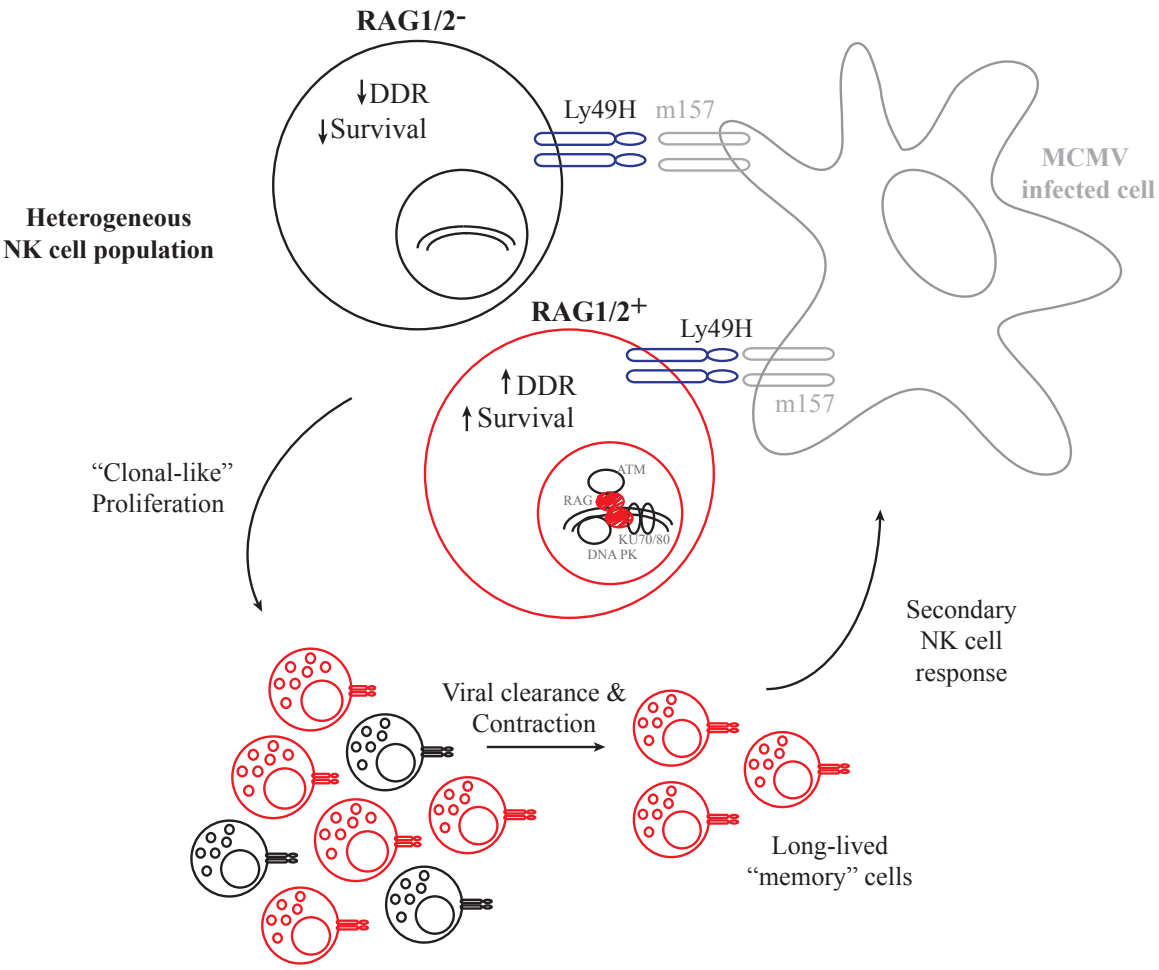

FIGURE 45 | RAG EXPRESSION DURING ONTOGENY DELINEATES FUNCTIONAL HETEROGENEITY AND CELLULAR FITNESS IN NK CELL SUBSETS.

NK cells from B6 mice expressing the activating Ly49H receptor will recognize MCMV-encoded m157 on infected cells, leading to activation and clonal proliferation of antigen-specific NK cells. History of RAG expression marks a subset of cells (red) that have enhanced DNA damage response (DDR) and survival following rapid proliferation. After the NK cell expansion phase and viral control, effector Ly49H$^+$ NK cells undergo a contraction phase resulting in long-lived memory NK cells, where only the NK cells that previously expressed RAG remain. These memory NK cells are able to mount a recall response when virus is re-encountered.

(RAG-competent) versus OT-1 x *Rag2*$^{-/-}$ mice (Hogquist et al., 1994), we found that RAG-deficient OT-1 cells possessed higher levels of genomic instability and decreased levels of essential DNA damage repair proteins (Karo et al., 2014). Radiation exposure resulted in delayed γ-H2AX foci resolution in RAG-deficient T cells, and virus infection revealed a reduced capacity for survival, compared to OT-1 cells (Karo et al., 2014). Further, ILCs with a history of RAG expression increased in the presence of *C. Rodentium* bacterial infection, suggesting a survival benefit from RAG expression during ontogeny. Thus, RAG expression during ontogeny endows both innate and adaptive lymphocytes with enhanced cellular fitness, suggesting this may be a general mechanism for producing immune memory in lymphocytes.

Heterogeneity within the NK cell compartment, as defined by prior RAG expression, may also exist in humans. Humans lacking RAG activity exhibit severe combined immunodeficiency (Schwarz et al., 1996) with the absence of T and B cells but possessing NK cells, whereas partial loss of RAG activity results in Omenn syndrome (Villa et al., 1998) or CID. Some of these patients with RAG mutations have been reported to be sensitive to radiation, presumably due to an inability to efficiently repair therapy-induced DNA DSBs (Blunt et al., 1996; Nicolas et al., 1998; Villa et al., 1998). Similarly, SCID and RAG-deficient mice are more sensitive to full body irradiation than WT mice (Biedermann et al., 1991), and it will be of interest to determine whether the greater morbidity and mortality of irradiated RAG-deficient mice is related to greater genomic instability. Given that NK cells from *SCID* mice behaved like the RAG-deficient cells, we predict that mice deficient in ATM or Ku80 would similarly exhibit

defects in the NK cell response to viral infection due to loss of gross genome integrity from accumulation of unrepaired DNA DSBs. In addition, because *SCID* NK cells exhibited an activated phenotype and enhanced cytotoxicity, like RAG-deficient NK cells, it is possible that DNA-PKcs is important for signaling downstream of RAG-mediated breaks early during NK cell ontogeny. Interestingly, the spectrum of RAG-related disorders often manifests in humans with autoimmunity and HCMV infection (de Villartay et al., 2005). Because virus-specific effector and memory NK cell responses to HCMV infection have recently been documented (Bjorkstrom et al., 2011; Della Chiesa et al., 2012; Foley et al., 2012b; Lopez-Verges et al., 2011), it will be of interest to determine whether lack of HCMV control in individuals with SCID or Omenn syndrome may be due to a decreased cellular fitness in NK cells responding to infection.

Recombination has also been linked to metabolic stress through AMP-activated kinase (AMPK), which plays roles in lymphocyte survival (Rolf et al., 2013; Tamas et al., 2006) and can directly phosphorylate and activate *Rag1* leading to initiation of RAG catalytic activity (Um et al., 2013). Studies suggest increasing cyclic-AMP (cAMP) levels also lead to increased V(D)J recombination (Menetski and Gellert, 1990), however this may be due to cAMP levels enhancing AMPK activation (Omar et al., 2009). The effect of metabolic output on V(D)J recombination and lymphocyte development has not been demonstrated in a physiological context; however, we hypothesize that metabolic deficiencies in NK cells that have not expressed RAG may result in their decreased fitness during proliferation, providing an additional mechanism for the functional heterogeneity observed following MCMV infection.

DDR leads to downstream transcriptional changes

DNA damage is generally assumed to be a harmful event, frequently associated with impaired cell survival or disease (Bednarski and Sleckman, 2012; Sherman et al., 2011). However, a growing body of evidence suggests that focal DNA breaks constitute a mechanism for normal cellular maturation, differentiation, and function. The observations delineated in this thesis demonstrate that enhanced DDR is necessary for immune cell "fitness" in response to episodes of robust proliferation. These data suggest DNA break and repair mediated by RAG recombinase might be essential for the optimal function of NK cells, T cells, and ILCs.

Recent studies support the idea that controlled DNA damage during cellular development acts as a novel mechanism that propagates the transcription and translation of proteins and factors that have important consequences for normal development and differentiation (Bednarski et al., 2012; Bredemeyer et al., 2008; Sherman et al., 2010). In pre-B cells, RAG-mediated DSBs induce expression of receptors important for lymphocyte egress from the bone marrow and promote homing to secondary lymphoid organs (Bredemeyer et al., 2008; Schwab and Cyster, 2007). Thus, the RAG-mediated expression of these receptors are thought to prevent pre-B cells from prematurely exiting the bone marrow before complete rearrangement of BCRs. In another study (Larsen et al., 2010b), caspase-3 mediated DNA damage leads to muscle cell differentiation by inducing expression of non-apoptotic genetic pathways. These studies begin to highlight the potential functional

consequence of DNA DSBs beyond being detrimental role to cell survival. In fact, downstream DSB-mediated transcriptional events may be necessary for the productive maturation of specific cell subsets, and support that idea that DDR by programmed DSBs may "imprint" lymphocyte functions long after the breakpoint has been repaired.

RAG-mediated DSBs have been shown to activate the canonical NF-kB pathway (Miyamoto, 2011; Wu et al., 2006), leading to the expression of several pro-survival factors in response to genotoxic DSBs (Elkon et al., 2005; Rashi-Elkeles et al., 2006; Wu et al., 2006). Approximately half of the ATM-dependent gene expression changes that occur in response to RAG DSBs require NF-kB (Bredemeyer et al., 2008). For example, activation of NF-kB by RAG DSBs leads to the upregulation of including *Pim2*, a constitutively active anti-apoptotic serine-threonine kinase, shown to be important for lymphocyte proliferation and survival (Amaravadi and Thompson, 2005; Bredemeyer et al., 2008; Malin et al., 2010; Nawijn et al., 2011). *Pim2* expression is increased in an ATM-dependent manner in response to RAG DSBs (Bednarski et al., 2012; Bredemeyer et al., 2008). Upregulation of *Pim2* maintains phosphorylation and inactivation of BAD, which antagonizes the pro-apoptotic function of BAX, promoting increased levels of Bcl-2 and associated cell survival (Bednarski et al., 2012; Youle and Strasser, 2008). In these studies, upregulation of *Pim2* by RAG DSBs results in reduced proliferation of IL-7 stimulated pre-B cells (Bednarski et al., 2012) by enforcing the G1 to S checkpoint and inhibiting the proliferation of pre-B cells with unrepaired DSBs (Bednarski and Sleckman, 2012; Domen et al., 1993; Mikkers et al., 2004). *Pim2* is not sufficient to block proliferation, but maintains cell cycle arrest due to coordination with other DSB-

dependent signals (Bednarski et al., 2012). In these cells, expression of *Pim2* promotes genomic stability by preventing unrepaired breaks to enter cell cycle resulting in further chromosomal aberrations during replication. Therefore, analysis of *Pim2* levels and cell cycle checkpoints in NK cells may provide insight to a mechanism whereby RAG-mediated breaks suppress aberrant cell cycle progression during development.

The impact of RAG-deficiency on cell cycle progression is also of interested because many DSBs are generated during normal DNA synthesis and elicit a DDR response that is different than RAG-mediated breaks. Specifically, RAG-mediated breaks occur only in G1 phase cells during development and are dependent on ATM, while replication-associated breaks occur during cell cycle/S phase throughout the life of the cell and are dependent on ATR signaling (Shiloh, 2003). Therefore, the intrinsic differences in these two DDR programs may be due to the cellular environment during cell cycle. These breaks may have unique features (e.g. structure, chromatin modulation) that initiate a specific transcriptional response. It is of interest to further determine differences in these two types of DDR responses and investigate how RAG-deficiency influences normal DNA repair during DNA replication. A recent study showed that induction of genotoxic DSBs lead to the nuclear export of RAG2 and localization to centrosome to stall cell cycle progression until DNA damage was repaired (Rodgers et al., 2015). It would be interesting to determine whether the increased level of DSBs at steady state in NK cells leads to changes in RAG2 localization. These observations suggest an additional mechanism for RAG2-mediated maintenance of lymphocyte genomic stability.

DNA damage programs that inhibit cell differentiation

NK cells that have previously expressed RAG proteins during ontogeny are less terminally differentiated, suggesting RAG-mediated DSBs may be inhibiting rapid cell maturation and promote a genetic program that is important for processes beyond canonical DDRs. This hypothesis is supported by observations in myoblasts, melanocyte stem cells (MSC), and hematopoietic stem cells (HSC), where DNA damage response may be affecting cell differentiation (Inomata et al., 2009; Puri et al., 2002; Rossi et al., 2007).

As myogenic precursor cells (myoblasts) differentiate into mature myotubes, they irreversible withdraw from cell cycle (Walsh and Perlman, 1997). Therefore, cell cycle arrest (e.g. due to genotoxic stress) could result in the unwanted arrest and terminal differentiation of aberrant cells. To combat this C2C12 myoblasts undergo cell-cycle arrest and block in differentiation in the presence of genotoxic stress, due to a DDR-regulated differentiation checkpoint during muscle differentiation (Puri et al., 2002). This block is reversible and can be overcome following the repair of damaged DNA. Furthermore, DNA DSB-initiated ATM signaling maintains MSCs within the stem cell niche and blocks differentiation into mature melanocytes (Inomata et al., 2009). Hair greying, an obvious sign of aging, results from the loss of melanocyte 'stemness' in subcutaneous hair follicles over time (Nishimura et al., 2005). DNA DSBs induce aberrant differentiation of MSCs into mature melanocytes result in gray hair (Nishimura et al., 2005). Induced differentiation is further exacerbated by the targeted deletion of *Atm*

(Nishimura et al., 2005). These results indicate that increased frequencies of DSBs in the absence of efficient repair promote the unwanted differentiation of MSCs and suggest DDR, namely ATM, is required to maintain MSC 'stemness' and quality.

It is also thought that DNA DSB accumulation over time leads to the functional decline of HSCs during aging. HSCs from DDR-deficient mice show significantly higher apoptosis and decreased proliferation, suggested that proper repair of DNA damage is required for efficient HSC renewal and function (Rossi et al., 2007). In the absence of ATM, ROS-induced DNA damage leads to the loss of self-renewal capacity and exhaustion of the HSC pool. Therefore, while physiological frequencies of DSBs can be handled by developing HSCs (Ito et al., 2006), loss of DDR and increased DSBs lead to impaired DNA repair and depletion of the HSC reconstitution and differentiation capacity (Sherman et al., 2011). Interestingly, aging HSCs exhibit a differentiation bias favoring the myeloid lineage with a concomitant decrease in lymphoid lineage potential (Rossi et al., 2005). Whether this aging bias is due to accumulation of DNA damage over time remains to be determined.

Overall, these studies suggest a protective function for DDR in stem and progenitor cells, similar to that observed in NK cells that have expressed RAG, in order to promote normal maturation and prevent the propagation of genetic errors. Additional studies are required to determine the interactions between DDR and mechanisms that block differentiation in these cells.

DNA damage changes the epigenome

Transcriptional activation is closely linked to alteration in chromatin structure and function. Chromatin modifications on histones (e.g. methylation and acetylation) can lead dynamic changes in expression and are able to persist through cell division, thus allowing a cell to 'remember' its transcriptional profile throughout development (Zediak et al., 2011). Therefore, changes in RAG expression during ontogeny in NK cells may result in changes in the epigenetic landscape thereby "imprinting" changes in these cells seen has functional diversity in the periphery.

The accessibility hypothesis, originally presented by Fred Alt in 1985, suggests that V(D)J recombination might be regulated through changes in the accessibility of the chromatin-embedded RSSs to the recombinase (Krangel, 2003). Therefore, recombination-active V(D)J loci should be associated with open and "transcriptionally active" chromatin confirmation, while inactive loci are associated with closed chromatin. In agreement with this, it has been shown that the RAG2 PHD C-terminal domain specifically recognizes H3K4me3 (a marker of transcriptional activation (Ji et al., 2010; Liu et al., 2007; Matthews et al., 2007)) throughout the genome and recruits the RAG complex. Thus, mutations that abrogate the ability of RAG2 to binding to H3K4m3 severely impaired V(D)J recombination (Liu et al., 2007; Matthews et al., 2007). The SWI/SNF ATP-dependent chromatin remodeling complex was also shown to be required to alter the nucleosome structure at IgH locus (Osipovich et al., 2009), providing direct

evidence that chromatin regulates RAG activity. Whether chromatin remodeling and histone modifications occur in NK cells due to RAG expression remains to be determined. Interestingly, we observed differential H3K4me3 expression within the *Chek2* promoter in NK cells with a history of RAG expression, suggesting RAG may "imprint" DNA histone modifications at sites lacking RSS sequences (e.g. DDR proteins). Therefore, further epigenetic analysis of regulatory regions surrounding DNA damage repair genes in WT compared to RAG-deficient NK cells might provide mechanistic insights into how damage response protein levels are modulated in an individual resting lymphocyte.

The ability of RAG2 (and potentially RAG1) to bind to transcriptionally active sites outside of antigen receptor loci raises questions about specificity and fidelity of V(D)J recombination. There exists evidence that suggests RAG-mediated cleavage may be occurring at these sites contributing to lymphoma-associated genome alterations (Gladdy et al., 2003; Lieber et al., 2006; Mills et al., 2003). The data from our group and others suggest these ectopic breaks might be evolutionarily conserved in order to create heterogeneity among cell types. Many questions remain unanswered. Are these RAG-mediated DNA break/repair events controlled or stochastic? How and when do the RAG proteins create DSBs at these non-antigen receptor sites in the genome? Finally, what additional mechanisms or other molecular chaperones exist to suppress (or promote) this ectopic DNA damage leading to differential cellular fates?

Larsen et al. demonstrated that caspase 3 promotion of DNA damage by caspase activated DNase (CAD) is essential for myoblast differentiation, and speculate this process is dependent on chromatin remodeling proteins (Larsen et al., 2010b). Specifically, the ste20-like kinase MST1 participates in this process by directly phosphorylating histone H2B leading to chromatin compaction and apoptosis (Cheung et al., 2003; Fernando et al., 2002; Graves et al., 1998). This process may be furthered altered by association with histone H1, which increases the nuclease activity resulting in differentiation inducing DSBs (Widlak et al., 2005). Interestingly, global DNA demethylation is observed in skeletal muscle differentiation concurrent to the period of CAD activation (Jost et al., 2001). These data show examples (outside of the RAG recombinase) where DNA DSBs may be associated with changes in epigenetics resulting in downstream regulation of gene expression. Further examination of epigenetic changes that occur at these sites will provide insight into how DSB and repair is utilized to regulate gene expression and differentiation.

During V(D)J recombination, the induction of RAG-mediated DSBs on one allele, during lymphocyte development, halts the introduction of further breaks on the second allele through action of ATM. Specifically, ATM is recruited to the breakpoint in the PCC and acts *in trans* to reposition the other (non-broken) allele into pericentromeric heterochromatin (Chaumeil and Skok, 2013). Transient formation of this repressive heterochromatin environment leads to the release of RAG on the unbroken allele while the broken allele is repaired, therefore facilitating allelic exclusion and protecting against aberrant translocation (Chaumeil and Skok, 2013). ATM-deficiency has also been shown

to orchestrate premature release of RAG-generated DSBs from PCC leading to alternative end joining, leading to chromosome aberrations (Bredemeyer et al., 2006; Callen et al., 2007). ATM-deficiency exacerbates these phenotypes due to downstream changes in transcription signatures (discussed above). Therefore, reduced levels of ATM in RAG-deficient NK cells may further lead to the increase in genomic stability seen by disrupting the process of allelic exclusion and PCC stability.

Studies have also suggested that histone modifications play a functional role in memory T cell development (Araki et al., 2008; Nakata et al., 2010; Northrop et al., 2006; Yamashita et al., 2006). Specifically, failure to differentiate into memory T cells is associated with lack of histone acetylation at the *Ifng* locus in CD8+ T cells (Araki et al., 2008). Histone methylation has been shown to be important in CD4+ T cell differentiation (Nakata et al., 2010; Yamashita et al., 2006), but data supporting a role in CD8+ T cell memory are lacking. Further, differentiation of naïve T cells into SLEC or MPEC may depend on heritable epigenetic marks that are established during the effector phase and persist into memory. It will be of interest to compare these different T cell and NK cell subsets (e.g. with or without RAG expression) to determine the role of "epigenetic memory" in immunological memory and the potential role RAG plays in maintaining these changes.

DNA methylation at cytosine residues occurs within high-density CpG regions in promoters and enhancers, called "CpG islands", and is repressive to transcription.

Therefore, methylation is low when genes are active, but increases when genes are repressed (Meissner et al., 2008; Weber and Schubeler, 2007). Recent studies have shown mice lacking the maintenance methyltransferase Dnmt1 or the methyl DNA binding protein MDB2 had reduced number of memory T cells and an altered pattern of memory differentiation (Chappell et al., 2006; Kersh, 2006), suggesting DNA methylation may be involved in normal memory CD8+ T cell differentiation. Given the important role for histone marks and DNA methylation in T cell memory, defects in setting up or maintaining epigenetic marks could result in memory dysfunction. Therefore changes in the epigenetic landscape may result in the cell survival and longevity defects observed in RAG-deficient NK cells.

The study of epigenetics has begun to ascertain and clarify the regulated recruitment of RAG recombinase to sites within the genome due to gene accessibility. It is possible that the RAG recombinase plays a regulatory function during ontogeny resulting in changes in transcriptional activity, histone modifications, or chromatin structure of non-antigen receptor loci in developing adaptive and innate lymphocytes. A more thorough investigation of these control mechanisms will disclose new information that may elucidate unforeseen roles for RAG outside of recombination.

Summary

Adaptive lymphocytes have long been known to require RAG-mediated DNA DSBs for development of functional antigen receptor genes. The results within this thesis suggest that controlled DNA breaks are also required for the development and long-lived "fitness" of innate lymphocytes, and that RAG is a major player in these events. The sum of these results indicate that RAG-mediated DSBs outside of canonical V(D)J recombination in developing lymphocytes results in the unexpected functional heterogeneity within NK cell populations, and RAG-generated DNA breaks may be regulating processes that impact their ultimate survival as mature cells. From these studies a more general paradigm is emerging where activation of the DNA damage response by DSBs generated during timed cell processes (such as transient RAG expression and activity in lymphocyte progenitors) regulate a multitude of cell-type specific programs. This endonuclease-mediated DDR mechanism may be dictating the genomic integrity and fitness of select cells within the total adaptive and innate lymphocyte populations, and ensuring the longevity of specific lymphocyte subsets during periods of rapid proliferation or stress during pathogen invasion. It remains to be determined whether this represents an evolutionary mechanism that is tolerated despite risks of genomic instability, or whether it represents a risk-free utilization of RAG endonuclease activity outside of V(D)J recombination.

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