Replication stress in activated human NK cells induces sensitivity to apoptosis

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Abstract

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Natural killer cells are innate immune effectors that kill virally infected or malignant cells. Natural killer cell deficiency (NKD) occurs when NK cell development or function are impaired, and individuals with NKD are susceptible to severe and recurrent viral infections. Several gene deficiencies result in NKD, including variants in MCM4, GINS1, MCM10 and GINS4, which are components of the CDC45-MCM-GINS (CMG) helicase. The CMG helicase unwinds DNA during replication and is expressed in any actively proliferating cell. NK cells are more strongly impacted by mutational deficiencies in helicase proteins than other lymphocytes, though the mechanisms underlying this susceptibility are not completely understood. NK cells from individuals with NKD as a result of helicase deficiency have increased DNA damage, cell cycle arrest, and replication stress. We found that activated NK cells undergo apoptosis and autophagy in response to this stress, unlike activated T cells. We also identified a patient with a damaging variant in CDC45 to further support these findings of the effects of replication stress on NK cells. This individual, due to broader involvement of the immune system, requires a wider definition of natural killer cell disease, termed NK IEI. However, this CDC45-deficient individual’s cells display disrupted cell cycle, increased DNA damage and replication stress, with upregulation of apoptosis genes in NK cells. These findings show that sensitivity to replication stress affects human NK cell survival and function and can contribute to NK cell deficiency and human disease.
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Finally, I would like to thank the Institute of Human Nutrition and Columbia University as a whole, as well as my friends and family for supporting me unconditionally as I pursued my dreams.
Dedication

For my husband Michael, I couldn’t have done this without you.

For Charlie and Grace, you are my sunshine.
Chapter 1: Unwinding the role of the CMG helicase in Inborn Errors of Immunity

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Key words: NK cells, cytotoxicity, development, CMG helicase, Inborn Errors of Immunity


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Introduction

Natural killer cells (NK) are critical components of the innate immune system, defined as non-T cell, non-B cell, CD56^+CD3^- large granular lymphocytes (3). Within peripheral blood mononuclear cells (PBMCs), NK cells comprise between 5 to 20% of lymphocytes, with about 90% of these being CD56^{dim}, more mature cytotoxic effectors and the remaining 10% encompassed by CD56^{bright}, less mature cytokine producers (4-6). NK cells arise in the bone marrow from CD34^+ hematopoietic precursors and ultimately develop through the common lymphoid progenitor (CLP) lineage (7). NK cells occupy an immunological niche by killing virally infected and malignant cells in a contact-dependent manner without antigen restriction (8, 9). Formation of the immune synapse (IS) consists of several critical steps that ultimately result in apoptosis of the target cell. Initiation stage begins with the reorganization of the plasma membrane to allow for lipid raft assembly at the IS and serve as a physical platform for aggregating activating receptors (10, 11). Initiation stage also includes the ligation of activating receptors such as NKG2D, followed by downstream activation signaling through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (12-14). Distribution of lipids is critical for intra- and intercellular signaling, organellar structure and plasma membrane functions (15, 16). In a positive feedback loop, activation of NK cells results in lipid rafts accumulating at the synapse to facilitate signaling for cytotoxicity by sequestering NK activating receptors such as CD2 and NKG2D to the IS (11). Next, during the effector stage, vital physical rearrangements occur, including accumulation of filamentous (F)-actin at the synapse, the microtubule organizing center (MTOC) moving towards the immune synapse, lytic granules containing the effector molecules perforin and granzyme polarizing towards the MTOC and lytic granules released across the synapse to trigger
apoptosis in the target cell (9). Finally, termination consists of the programmed death of the target cell and detachment of the effector cell, following which, NK cells can go on go serially kill additional targets or enter into a state of exhaustion (8, 9). It should be noted that disruption at any point in this cascade of events results in impaired killing of infected target cells. Additionally, NK cells may activate death receptors on the surface of the target cell, including TNF-related apoptosis-inducing ligand (TRAIL) and Fas Ligand (FasL), resulting in the caspase-cascade towards apoptosis (9, 17). The wide variety and distinctive activities that NK cells participate in point to the important role they play in human biology.

Cell cycle and proliferation are key activities for tissue maintenance, bone marrow niche turnover, immunological response to infections, and other biological functions. The role of proliferation in immunology allows effector cell types to quickly expand in response to a threat (18, 19). Regulation of cell cycle progression plays a critical role in proliferation, as it is guarded by cell cycle checkpoint proteins. At the cellular level, cell cycle checkpoints can be activated to prevent the cell from progressing for a variety of reasons, such as a lack of nutrients, DNA damage, or other external signals (20, 21). These checkpoints are primarily regulated by cyclins and their counterpart cyclin-dependent kinases (CDKs), as well as downstream transcription factors including E2F1-6 (20) which impact gene transcription. Thus, many proteins are differentially expressed during the cell cycle, which can also control cytotoxic function. Thus, the significance of studying NK cell cytotoxic function and overall biological processes including those underlying cell cycle and proliferation, and cytokine responses highlights the need for a better understanding of NKD.

Inborn errors of immunity (IEI) occur as the result of inherited monogenic variants that affect human immune function and lead to immunological disease (22). More than 500 individual
genes have been ascribed to various immune conditions that result from IEI, including combined immunodeficiencies, immune dysregulation, and autoinflammatory conditions. Natural killer cell deficiency (NKD) accounts for a small subset of IEI where the primary immune lineage that is affected are natural killer cells (3). NKD presents with a lack of NK cells or a lack of cytotoxic function and is accompanied by severe recurrent viral infection (3). Viruses in the Herpesviridae family, including varicella zoster virus (VZV), human cytomegalovirus (HCMV), and Epstein-Barr virus (EBV) among others, are particularly susceptible to NK cell-mediated immunity (23). It is thought that herpes viruses act to evade cytotoxic T lymphocyte responses primarily by downregulating HLA-I expression in infected host cells, which consequently renders them susceptible to killing by natural killer cells (23, 24). Alternatively, other viruses such as influenza and parainfluenza can be recognized by binding to Natural Cytotoxicity Receptors (NCRs) like haemagglutinin or haemagglutinin-neuraminidase, respectively, to NKp46 on NK cells (25). Two classes of NKD have been described: functional (fNKD) and classical (cNKD). The former presents with normal NK cell numbers in peripheral blood but exhibit decreased effector function, while the latter may affect NK cell development, survival, and maturity resulting in <1% NK cells (3, 24). It should also be noted that cNKD could preferentially affect the frequency of certain NK cell subsets.

While there are few monogenic causes of NKD, an unexpected source of NKD is variants in the CMG helicase that forms the DNA replisome (26-31). Despite the ubiquitous expression of, and requirement for, this complex in cellular function, the sole or primary immune defect in patients with these variants is frequently within the NK cell subset. The identification of these variants has provided new insight into the requirements for NK cell differentiation and homeostasis and led to a closer examination of the differential requirements for innate lymphocyte development.
and activation. Here, we will review the latest findings on the role of the CMG helicase in human immune cell function, primarily informed by studying variants associated with NKD.

1.1 NK cell memory

Despite their lack of germline-encoded antigen specific receptors, NK cells can also have features of immune memory, including rapid expansion upon secondary infection, and epigenetic and transcriptional changes that lead to the generation of long-lived memory cells (32-35). Human cytomegalovirus (HCMV) infection can induce the production of memory NK cells through NKG2C-HLA-E interactions (32, 36-38). Other viruses, such as EBV and HIV, induce a subset of long-lived memory NK cells with increased effector functions upon recall (32). Adaptive NK cells can be modeled in vitro using cytokines, termed cytokine induced memory-like (CIML) NK cells, which occurs following stimulation with IL-15, IL-18, and IL-12 and results in NK cell activation of mouse and primary human NK cells (39-41). Cytokines are found throughout the immune system to act in concert or alone to produce anti-inflammatory or pro-inflammatory responses. In terms of NK cells, cytokines are critical to development and maturation, proliferation potential, and cytotoxic function (7, 42, 43). IL-15 is produced by a variety of cells, including monocytes, DCs, epithelial and stromal cells, as well as fibroblasts, to generate an array of responses in NK cells and CD8+ T cells (29). In mature NK cells, these responses are two-fold; increased metabolism to result in increased proliferation (43, 44) and upregulation of various molecules involved in cytotoxicity, such as NKG2D, TRAIL, FasL and perforin (45). These cytokine responses are finely tuned, however, as continuous IL-15 stimulation can induce a state of exhaustion of NK cells, with decreased metabolism and proliferation, decreased cytotoxic function, and a gene expression signature associate with cell cycle arrest (46). Cytokine signaling is propagated through the JAK/STAT pathway, where
specifically STAT5 is responsible for IL-15 signaling. Thus, IL-15 is critical for NK cell maturation and survival, while IL-18 and IL-12 are produced by antigen presenting cells (APCs) in response to viral infection (47, 48). CIML NK cells proliferate and upregulate cytotoxic responses upon stimulation and can serve as a useful model of primary NK cell activation during viral infection. In vivo, these activated NK cells exhibit heightened responses against infections and cancerous target cells (40, 49, 50).

1.2 The CMG Helicase

Faithful DNA replication during S phase occurs only once per cell cycle and is critical for maintaining genomic stability. The CMG helicase is composed of 11 subunits of the CDC45-MCM-GINS complexes (51) and is responsible for unwinding double-stranded DNA and recruiting polymerases for DNA replication (Fig 1.1). CDT1, CDC6, and the double hexamer MCM2-7 complex are recruited to the origin recognition complex (ORC) during G1 phase of the cell cycle; CDC45 and GINS1-4 are subsequently recruited to complete the formation of the CMG helicase.

**Figure 1.1 CMG Helicase during DNA replication.** The CMG helicase primarily functions in S phase to trigger origin firing (1) and DNA unwinding (2) to facilitate DNA replication with the recruitment of polymerase (3). An intact helicase maintains replication fork and genomic stability, participates in DNA damage signaling, and is critical for DNA replication to occur (4).
helicase. The MCM complex is the catalytic activity center of the CMG helicase as each individual subunit is an ATPase (52). The intact helicase can trigger origin firing and unwinding of the DNA double helix, allowing DNA polymerase to begin priming the DNA (51, 53, 54). The engagement of MCM10 and other factors including PCNA facilitates elongation of the replication fork and aids in overall stability during replication (55-57).

Assembly of the CMG helicase is dynamic and involves the assistance of accessory proteins for proper function and stability. These accessory proteins could also potentially be involved in the development of NKD (Fig 1.2). CMG helicase function is required in any actively proliferating cell, and the reason why NK cells are seemingly particularly susceptible to damaging variants has not been elucidated.

**Figure 1.2 CMG helicase assembly.** A model of CMG helicase assembly during G1/S phase. Subunits shown in red font are helicase complex members and related proteins that have been implicated in NKD.
The CMG helicase, both as individual components and as a functional complex, is highly regulated during DNA replication and, by extension, the cell cycle. Multiple kinases, including Cyclin A-Cdk2 and Dbf4-Cdc7, function to activate the helicase at entry to S phase \((58, 59)\). Another form of regulation lies with CDC45 protein expression, which in mammalian cells has been shown to be the rate-limiting factor in DNA replication \((60, 61)\). Further regulation of the helicase complex is found through the amount of protein actively used. It is thought that an excess of MCM protein is loaded onto chromatin while lacking colocalization with replication forks to ensure ample coverage of origins of replication, a paradigm commonly called the MCM paradox \((62)\). However, recent work has highlighted a possible role for this excess of MCM protein in replication fork speed and in response to replication stress \((63)\). Thus, tight control of the CMG helicase during canonical DNA replication is critical to maintaining genome stability, accurate replication of the genome, and cell cycle progression.

1.3 Noncanonical roles for helicase proteins

In addition to the canonical role that helicase proteins play as part of a functional complex in DNA replication, several CMG proteins have auxiliary roles in other cellular functions unrelated to replication. CDC45, for example, binds to single-stranded DNA (ssDNA) \((64)\) and single-strand/double-strand DNA junction sites \((65)\). CDC45 is also responsible for loading replication protein A (RPA) onto nascent ssDNA at the replication fork through binding to the RPA70A subdomain \((66)\). In yeast, Cdc45 is involved in checkpoint signaling during fork stalling by binding with Rad53 \((67)\). As such, CDC45 may play an additional specific role in the sensing of ssDNA and signaling for the DNA damage response.

MCM2-7 proteins likewise can have supplementary roles separate from replication \((68)\). MCM proteins, particularly MCM2, have been implicated in transcriptional control, and blocking
MCM2 activity with antibodies inhibits RNA polymerase II (Pol II) transcriptional machinery (69). MCM5 has also been found to bind directly to the transcription factor STAT1 to increase transcription activation during interferon-λ stimulation, an important avenue of MCM function in immunity (70, 71). Mcm7 likewise has been shown to bind with proteins critical to cellular functions, namely retinoblastoma tumor suppressor protein (Rb), which regulates cell cycle and proliferation through E2F (72) and E6 oncoprotein, likely contributing to the development of cancer (73). Finally, MCM proteins may play a role in chromatin regulation and maintenance, including binding of Mcm2 to histone H3 (74) and MCM10 in partnership with MUS81 (31).

The GINS complex, likely through helicase activity in replication and proliferation, is also involved in mammalian development of the nervous system and early embryogenesis (75, 76). Gins2 is required for zebrafish development through interaction with Gins4 protein and promotes overall CMG helicase stability (77). Outside of CMG helicase activity, increased expression of Gins4 has been associated with advanced gastric cancer. Gins4 binds to and activates the Rac1/CDC42 complex, which is a signaling pathway related to cell proliferation and migration (78). Another divergent role for Gins is involvement in chromosomal alignment during mitosis (79). GINS4, also called Sld5, recruits PCM-1, and loss of Sld5 function leaves chromosomes susceptible to fragmentation.

### 1.4 Helicase variants and NKD

Work by our lab and others have defined variants in the eukaryotic DNA helicase complex, including MCM4, GINS1, MCM10, and GINS4, that result in NKD. Specifically, NKD due to helicase variants manifests as impaired NK cell differentiation and maturation leading to significant functional impairment and resulting viral infections in affected individuals (Table 1.1). At the cellular level, these mutations lead to impaired cell cycle progression, replication stress,
increased DNA damage and DNA damage responses and, ultimately, apoptosis (26-30) (Table 1.2). Programmed cell death, or apoptosis, is found in numerous immunological processes, such as contracting effector cell populations after infection and during lymphocyte development (80). In cases of MCM10 loss of function, for example, DNA damage is present via γH2AX, which immediately binds DNA upon detection of double-strand breaks (DSB) (27). The response to DSB can include activation of cell cycle checkpoints to induce cell cycle arrest followed by activation of the DNA damage response (DDR) pathway for repair, or cells may undergo apoptosis (81). In cases of severe DNA damage, the cell may be unable to adequately perform repairs and will undergo apoptosis (81-83). Indeed, increased apoptosis and failure to respond to proliferative cytokines has been described in MCM4-deficient NK cells (30). In light of the fact that helicase-deficient NK cells are in a state of cell cycle arrest with significant DNA damage, investigating the subsequent survival capacity through an apoptotic lens will illuminate the response to cytokine stimulation.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Number of patients</th>
<th>Clinical phenotype</th>
<th>Viral Infections</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MCM4</strong></td>
<td>6 (30, 84) + 8 (28)</td>
<td>Viral infection, short stature, adrenal insufficiency</td>
<td>P1 (84): EBV, HSV, VZV</td>
<td>(28, 30, 84)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P2 (84): respiratory infections, EBV, HSV</td>
<td></td>
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<td></td>
<td>P3 (84): EBV, HSV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P4 (84): respiratory infections, EBV, HSV, VZV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P2.11 (30): HSV, VZV</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>P6 (28): pneumonitis</td>
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<tr>
<td><strong>GINS1</strong></td>
<td>5</td>
<td>Viral infection, intrauterine growth retardation, neutropenia</td>
<td>P1 (29): CMV, chest infection</td>
<td>(29, 85, 86)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P2 (29): chest infection, rotavirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P3 (29): CMV, VZV, adenovirus, RSV, rotavirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P4 (29): VZV, HSV, chest infection</td>
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<td></td>
<td></td>
<td></td>
<td>P5 (29): VZV, influenza</td>
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<tr>
<td><strong>MCM10</strong></td>
<td>1</td>
<td>Viral infections</td>
<td>CMV</td>
<td>(27)</td>
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<td><strong>GINS4</strong></td>
<td>2</td>
<td>Viral infections, intrauterine growth restriction and growth delay, neutropenia</td>
<td>P1: BCG infection after vaccination, CMV, VZV, HSV</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P2: VZV, HSV</td>
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<td><strong>POLA1</strong></td>
<td>6</td>
<td>XLPDR, viral infections, intrauterine and postnatal growth retardation, hypogonadism</td>
<td>Respiratory infections</td>
<td>(87)</td>
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<td><strong>POLE2</strong></td>
<td>1</td>
<td>Respiratory viruses, BCG and Candida albicans infections, diphtheria and tetanus toxoid, combined immunodeficiency, hypothyroidism, facial dysmorphism</td>
<td>BCG infection after vaccination, respiratory infections</td>
<td>(88)</td>
</tr>
<tr>
<td>Gene</td>
<td>Report variant type</td>
<td>Variant effect (protein)</td>
<td>Ref</td>
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<td>--------</td>
<td>--------------------------------------</td>
<td>--------------------------</td>
<td>-----</td>
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<tr>
<td>MCM4</td>
<td>Homozygous c.70_71insG (frameshift → stop gain)</td>
<td>Truncated protein</td>
<td>(28, 30, 84)</td>
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<td>MCM10</td>
<td>Compound heterozygous c.1276C&gt;T p.R426C (missense); c.1744C&gt;T p.R582X (stop gain)</td>
<td>Partial loss of function Nonsense mediated decay</td>
<td>(27)</td>
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<td>POLA1</td>
<td>Hypomorphic X-linked g.24744696A&gt;G c.328G&gt;A</td>
<td>Unknown Hypomorphic</td>
<td>(87)</td>
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<td>POLE2</td>
<td>Homozygous splice-site c. 1074-1G&gt;T</td>
<td>Loss of function</td>
<td>(88)</td>
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<table>
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<tr>
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<th>Cell Cycle</th>
<th>Mouse Model</th>
<th>Reference</th>
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<tbody>
<tr>
<td>MCM4</td>
<td>↓ NK cell number and frequency ↓ CD56&lt;sup&gt;dim&lt;/sup&gt; NK cells ↓ CD57&lt;sup&gt;+&lt;/sup&gt; NK cells ↓ Perforin&lt;sup&gt;+&lt;/sup&gt; NK cells ↓ Cell proliferation ↑ Apoptosis (CD56&lt;sup&gt;bright&lt;/sup&gt; &amp; CD56&lt;sup&gt;dim&lt;/sup&gt; NK cells)</td>
<td>Fewer cells in G1/S phases, more cells in G2/M phases (patient SV40 fibroblasts)</td>
<td>Chaos3 (hypomorphic); Mcm4&lt;sup&gt;−/−&lt;/sup&gt; (embryonic lethal). ↓ NK cells No growth retardation Chromosomal instability</td>
<td>(28, 30, 89)</td>
</tr>
<tr>
<td>GINS1</td>
<td>↓ CD56&lt;sup&gt;bright&lt;/sup&gt; &amp; CD56&lt;sup&gt;dim&lt;/sup&gt; NK cells Unresponsive to cytokine stimulation</td>
<td>Fewer cells in G1 phase, more cells in G2/M phases (primary fibroblasts)</td>
<td>Gins1&lt;sup&gt;−/−&lt;/sup&gt; (embryonic lethal)</td>
<td>(29, 90)</td>
</tr>
<tr>
<td>MCM10</td>
<td>↓ NK cell number and frequency ↑ CD56&lt;sup&gt;bright&lt;/sup&gt; NK cells relative to CD56&lt;sup&gt;dim&lt;/sup&gt; ↑ doubling time (MCM10-KD cell line) ↑ γH2AX foci Normal NK cell cytotoxic function</td>
<td>S phase arrest (MCM10-KD NK cell line)</td>
<td>Mcm10&lt;sup&gt;−/−&lt;/sup&gt; (embryonic lethal)</td>
<td>(27, 91)</td>
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<td>Knockdown Effect</td>
<td>Observations</td>
<td>References</td>
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<tr>
<td><strong>GINS4</strong></td>
<td>↓ NK cell number and frequency</td>
<td>Mild mitotic exit defect (patient B cell lines)</td>
<td>(26, 76)</td>
<td></td>
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<tr>
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<td>↓ CD94&lt;sup&gt;+&lt;/sup&gt; NK cells</td>
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<tr>
<td></td>
<td>↓ NK cell cytotoxic function (K562 target cells)</td>
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<tr>
<td></td>
<td>↓ IFN-γ production</td>
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</tr>
<tr>
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<td>↓ survival when stimulated with PMA</td>
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<tr>
<td><strong>POLA1</strong></td>
<td>↓ NK cell number and frequency</td>
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<td>None</td>
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<td></td>
<td>↓ lymphocytes</td>
<td></td>
<td>(87)</td>
<td></td>
</tr>
<tr>
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<td>↓ CD56&lt;sup&gt;dim&lt;/sup&gt; NK cells</td>
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<td>↑ CD56&lt;sup&gt;bright&lt;/sup&gt; NK cells</td>
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*KD* knockdown, *PMA* phorbol 12-myristate 13-acetate
1.5 Human NK cell development

Insights into the specific effect of helicase variants on NK cells can be gained by careful study of human NK cell development. NK cell and innate lymphoid cell (NK/ILC) precursors are found in lymphoid tissues including fetal liver, bone marrow, thymus, and secondary lymphoid tissues (92-94). In the adult, NK cells are derived from bone marrow precursors and further mature in lymph nodes (92). Studies of the in vitro generation of NK cells has shown that cytotoxic NK cells are successfully generated from CD34+ precursor cells from cord blood, bone marrow, tonsil, and lymph node in the presence of cytokines (IL-3, IL-7, IL-15, SCF, and Flt3-L) and stromal cells (95-97).

Circulating human NK cells are commonly identified as CD3− CD56/16+ by flow cytometry. Although recent single cell and scRNA-seq analysis have revealed that NK cells are highly heterogeneous (98-101), the two major populations of peripheral blood NK cells are frequently defined as CD56brightCD16− (~10% of NK cells), and CD56dim CD16+ subsets (~90%) (102). CD56brightCD16− NK cells have lower basal cytotoxicity but secrete high amounts of IFN-γ in response to exogenous cytokines including IL-12 and IL-18, which are derived from activated dendritic cells and macrophages in the context of inflammation and infection. CD56bright CD16− NK cells also express cytokine receptors including CD25 (high-affinity IL-2Ra), CD117 (SCF receptor), and CD127 (IL-7Ra), which are not highly expressed on CD56dim NK cells. Cytotoxic function in CD56bright CD16− NK cells is decreased relative to CD56dim NK cells, but they can acquire cytotoxic function in response to IL-2 and IL-15 in concert with rapid proliferation (103). In contrast, CD56dim CD16+ NK cells have higher basal cytotoxic capacity, but their proliferation...
potential is limited except for the CD62L+ CD57− subset, which is considered a developmental intermediate between CD56bright and CD56dim (104).

In vivo and in vitro studies seem to demonstrate that CD56bright NK cells are precursors of CD56dim cells. CD56bright cells have a longer telomere length than CD56dim, and IL-2 or IL-15-activated CD56bright cells acquire CD16 and killer immunoglobulin-like receptors (KIR) during their proliferation (104, 105). The differentiation of CD56bright to CD56dim is also confirmed in humanized mice (106) as well as reconstituted NK cells in hematopoietic stem cell transplantation recipients (107, 108) and scRNA-seq studies (98, 109). While human NK cell precursors are thought to be continuously generated from bone marrow, secondary lymphoid tissues such as lymph nodes also have a role in the generation and differentiation of NK cells (110). Ultimately, the sites and homeostatic dynamics of human NK cell differentiation remain poorly understood.

1.6 NK cell kinetics and NKD

In addition to their different functions, the two major subsets of peripheral blood NK cell have different homeostatic turnover rates. In vivo kinetics studies showed that circulating NK cells more rapidly proliferate and die compared to T cells, even without infections (111). Further, immature CD56bright CD16− NK cells generated from bone marrow proliferate faster than CD56dim subsets (111). Even though CD56dim NK cells are less proliferative, a long-lived and self-renewing adaptive NK cell pool is consistently maintained (111). Therefore, an imbalanced CD56bright / CD56dim ratio, such as is often found in NKD, may not be due to increased CD56bright NK cells from a block in terminal maturation but can instead suggest that the long-lived adaptive NK cell pool is not stably differentiated from short-lived CD56bright NK cells.

In addition to inherited variants, de novo mutations can cause an NKD phenotype in adults. One such case is heterozygous GATA2 mutations which can cause the loss of circulating dendritic
cells, monocytes, B cells, and NK cells (112-116). While the cellular phenotype of GATA2 can be quite variable, the selective loss of the CD56\textsuperscript{bright} subset of NK cells is a highly conserved phenotype and, in some individuals with GATA2 deficiency, NKD is the predominant immune phenotype (117-119). This finding was confirmed in patients with paroxysmal nocturnal hemoglobinuria (PNH) (120). In this study, newly produced glycosyl-phosphatidylinositol (GPI)-negative immune cells from hematopoietic stem and progenitor cells with somatic PIGA mutations make chimerism with GPI-positive cells, and they are clearly discriminated by flow cytometry analysis of GPI expression. While T and B lymphocytes are maintained and dominantly GPI-positive, in the short-lived neutrophil population the GPI-negative ratio increases over time as they are continuously produced from the precursor cells in the bone marrow. Interestingly, the fraction of GPI-negative CD56\textsuperscript{bright} CD16\textsuperscript{−} NK cells correlate with neutrophils, which implies that turnover of this NK cell subset is rapid and that these cells are not differentiating into CD56\textsuperscript{dim} NK cells. In contrast, GPI-positive populations are dominant in CD56\textsuperscript{dim} NK cells, and the fraction is persistent over time compared with T and B cells, suggesting that these cells are long-lived. It may also be speculated that this impaired generation of mature NK cells, including CD56\textsuperscript{dim} and adaptive NK cell subsets, is what leaves individuals with NKD susceptible to recurrent viral infection. The study of human NK cells in the context of naturally occurring variations demonstrates different homeostatic properties and ontogenies of the CD56\textsuperscript{bright} and CD56\textsuperscript{dim} subsets and suggests differential mechanisms for the generation and maintenance of these subsets that can be further informed by considering the effect of helicase deficiencies on NK cells.

### 1.7 MCM4

MCM4 was the first helicase complex member associated with NKD when multiple families with a variant in MCM4 (c.71-1insG) were identified (85, 86). These individuals present with adrenal
insufficiency, growth retardation and developmental abnormalities, NKD, and recurrent viral infections such as herpes simplex virus (HSV) and varicella zoster virus (VZV). In 2012, another group published a clinical description of eight additional patients from an endogamous Irish traveler cohort with the same variant in MCM4 and a similar clinical phenotype (28). Gineau et al investigated the mechanisms underlying MCM4 deficiency by testing the variant in patient-derived fibroblasts and determined it results in a prematurely truncated protein at the N-terminal domain (30). However, cell extracts from patient fibroblasts showed interaction of truncated MCM4 with other MCM complex proteins as well as binding to chromatin, suggesting it could retain some function in activities critical to DNA replication, including initiation and elongation.

Further investigation into DNA replication found a decreased proportion of MCM4-deficient fibroblasts in G1 and S phase compared to controls with a concomitant increase of cells found in G2 and M phases (30). DNA content by propidium iodide labeling of patient fibroblasts was also higher than that of controls, indicating a disruption in the synchronization of replication and an overall cell cycle defect. This phenotype was amplified by aphidicolin treatment, which is an inhibitor of DNA replication by binding to polymerase-α. Assessment of genomic stability was also analyzed by calculating the average number of chromosome breaks in metaphase and demonstrating that patient fibroblasts had significantly more breaks. The addition of aphidicolin to cells with truncated MCM4 protein resulted in reduced DNA damage repair efficiency relative to those with full-length MCM4, collectively demonstrating that the truncated MCM4 variant leads to decreased cell cycle progression resulting in genomic instability and increased sensitivity to DNA damage.

Despite the ubiquitous function of the CMG complex in DNA replication and cell cycle, there was no impact on myeloid subsets or T cells in affected individuals, but a slight decrease in
CD27\(^{+}\)CD19\(^{+}\) memory B cells and a significant decrease in natural killer cell numbers was noted (30). Specifically, CD56\(^{\text{dim}}\) NK cells were decreased, resulting in an increased ratio of CD56\(^{\text{bright}}\) to CD56\(^{\text{dim}}\) NK cells and decreased proportions of perforin-positive NK cells and terminally mature CD57\(^{+}\) NK cells (30). CD56\(^{\text{bright}}\) NK cells did not proliferate in response to IL-2 or IL-15, which are cytokines required for NK cell maturation, proliferation, and survival. Patient CD56\(^{\text{bright}}\) NK cells had increased apoptosis that could be rescued by the addition of IL-2 and IL-15, while CD56\(^{\text{dim}}\) cells did not respond to stimulation. Notably, NK cells are also affected in the hypomorphic *Chaos3 (Mcm4)* mouse model, and this defect is found in addition to global genomic instability, defects in the adrenal gland, and severe growth failure (30). Gineau et al speculated that since MCM4 has higher expression in CD56\(^{\text{bright}}\) NK cells, a deficiency in this protein results in a block of terminal maturation into the CD56\(^{\text{dim}}\) subset through an accumulation of chromosomal damage during proliferation (30).

### 1.8 GINS1

The tetrameric GINS complex, including GINS1-4, is required for initiation and elongation of DNA replication by unwinding DNA and preferentially binding to single-stranded DNA (54, 121). Five individuals with a compound heterozygous mutation in *GINS1* have been described (29, 85, 86). Consistent with the MCM4 clinical phenotype, these individuals have growth restriction, susceptibility to viral infections, and low NK cell numbers in peripheral blood (29). Unlike the MCM4 patients, the five GINS1-deficient individuals do not have adrenal insufficiency, though chronic neutropenia is present, and two patients also had transient disruptions in T and B cells, particularly CD8\(^{+}\) T cells and memory B cells (85, 86). Cottineau et al further investigated the role that GINS1 plays in NKD (29). Importantly, GINS1 patients had low proportions of both CD56\(^{\text{bright}}\) and CD56\(^{\text{dim}}\) subsets, which did not respond to stimulation with IL-2 or IL-15, unlike the MCM4
patients (29). However, while both NK cell subsets were decreased, there was a greater relative decrease of CD56\textsuperscript{dim} NK cells, suggesting that a similar block in NK cell terminal maturation may also be present as in MCM4. In addition, certain ILC subsets were also decreased in these patients, suggesting that a common precursor may be affected.

GINS3 and GINS4 protein expression levels are also decreased in GINS1-patient derived cells, indicating that GINS complex stability and function are affected by loss of GINS1 protein expression (29). Additionally, Cottineau et al. showed that primary fibroblasts from patients exhibited abnormal nuclear morphology, including increased nuclear area and disproportionate misshapen nuclei, often with multi-lobes of varying sizes, indicating a failure to maintain genome integrity and signify mis-segregation of chromosomes. Further investigation into the cell cycle of primary fibroblasts showed increased percentages of cells in G2 and M phases for patient cells, with a concurrent decrease of cells in G1 phase. Patient cells were slow to re-enter cell cycle after synchronization, but eventually the proportion of cells in S phase was maintained. DNA fiber analysis determined that patient cells had disruptions in replication patterns, including decreased numbers of replication clusters and bidirectional forks as well as an increase in stalled replication forks. Replication fork speed was determined to be faster than controls, likely as a mechanism to compensate for the decreased number of active replication forks. Thus, in GINS1-variant cells, DNA replication can be initiated but not all replication origins are activated or maintained. In addition, patient fibroblasts proliferate slower than controls and have a senescent phenotype. DNA damage in response to hydroxyurea was higher in patient E6/E7-fibroblasts as indicated by γH2AX and 53BP1 foci (29). While the DNA damage response through CHK1 and RPA phosphorylation was decreased, CHK2 phosphorylation was normal, indicating that the ATR signaling pathway is affected by GINS1 deficiency, not the ATM signaling pathway. As in
the case of MCM4 deficiency, selective NKD is suspected to be the result of negatively impacted NK cell survival. It is likely that the phenotypes of impaired cell cycle progression, replication dynamics, and DNA damage contribute to decreased survival of specific NK cell subsets and neutrophils, although the basis for neutropenia found in GINS1, but not MCM4 deficiency, is not understood.

1.9 MCM10

Several additional components are necessary for optimal function of the CMG helicase. MCM10, while distinct from the MCM2-7 helicase, is a highly conserved component required for assembly of the CMG helicase (53) and binding to both single- and double-stranded DNA during origin unwinding (57). Importantly, MCM10 is also essential for the recruitment of polymerase-α and its association with chromatin (57), thus MCM10 is necessary for elongation of DNA synthesis as well as initiation.

Like MCM4 and GINS1, a compound heterozygous variant in MCM10 was determined to be causative of NKD in a single patient with fatal cytomegalovirus (CMV) infection (27) and decreased circulating NK cells in peripheral blood with an increased percentage of the CD56bright cells. While MCM10 protein expression wasn’t affected, patient variants displayed increased chromatin association indicative of a disruption in cell cycle and impaired function of MCM10 protein (27). Like the irregular nuclear morphology of MCM4 and GINS1 variant cells, MCM10 patient fibroblasts displayed increased nuclear area and increased frequencies of γH2AX foci. An increased frequency of patient cells in S phase further indicated that MCM10 variants have a cellular phenotype of replication stress and disrupted cell cycle dynamics. The cell cycle defect was maintained in NK cell lines, with an increased percentage of cells found in S phase as well as an increased doubling time. Consistent with the patient-derived fibroblasts and other published
helicase mutations, MCM10 knockdown in the NK92 NK cell line led to more γH2AX foci at baseline and in response to irradiation. It should also be noted that cytotoxic function was unaffected in these cells, indicating that MCM10 does not play a direct role in killing functions, but likely only in maturation and proliferative responses of NK cells. To investigate the role of MCM10 in development of natural killer cells, MCM10 was knocked down in CD34+ precursor cells from a healthy donor using shRNA. When cultured to make in vitro NK cells, the MCM10 deficient precursors led to an increased proportion of immature subsets with a concurrent decrease in CD16+ mature NK cells. NSG mice were injected with CD34+ precursors that were produced from patient-derived fibroblasts reprogrammed into induced pluripotent stem cells (iPSCs). This mouse model reconstituted peripheral blood and splenic NK cells, with increased CD56bright cells and increased γH2AX foci. Using both in vitro models and in vivo reconstitution, the MCM10 protein was determined to be a critical component required for NK cell maturation and extended the phenotype found in core CMG complex members to other regulators of cell cycle and genomic stability.

1.10 GINS4

GINS4 has recently been identified as the fourth monogenic cause of NKD related to the CMG helicase (26). GINS4 is component of the GINS tetrameric complex and thus plays a similar role to the GINS1 protein in initiation and elongation during DNA replication. In Drosophila, GINS4, also known as Sld5, interacts with GINS1 and GINS2 as well as MCM10 to preserve genomic integrity (122). In humans, GINS4, which is structurally similar to GINS1, functions as part of the GINS complex to recruit additional components required for DNA replication, including CDC45 and polymerases (123). The absence of Sld5 expression causes cell cycle delays in M and S phase
in *Drosophila* cells, while overexpression of this protein has been associated with human colorectal (124), lung (125), and bladder (126) cancers.

Low numbers of NK cells were detected in two siblings with inherited biallelic *GINS4* variants, with a higher frequency of CD56\textsuperscript{bright} cells present in peripheral blood (26). There was also noted to be intrauterine growth restriction and susceptibility to viral infection, a phenotype that resembles other helicase variants that cause NKD. Like the GINS1 patients, GINS4 individuals had neutropenia that responded productively to granulocyte colony-stimulating factor (G-CSF), while other immune subsets such as T and B cells were normal, including γδ T cells, and mucosal-associated invariant T (MAIT) cells. Patients had decreased NK cell cytotoxic function in a standard four-hour chromium release experiment against K562 tumor cells and NK cell cytotoxic function was not rescued by exogenous IL-2. Like the MCM10 study, GINS4 was knocked down in CD34\textsuperscript{+} precursors to examine the effect of GINS4 protein loss on the development of mature NK cells. Mature NK cells were significantly decreased compared to controls after in vitro differentiation.

The two siblings express ~15% of GINS4 protein relative to unaffected individuals, which affects assembly of the GINS complex as well as expression levels of other GINS proteins, namely GINS1 and GINS3. Interestingly, there were no significant changes to the cell cycle profile of patient-derived B cell lines, yet proliferation studies of patient-derived cells showed they were slower to re-enter cell cycle (26). Gene expression analysis further confirmed no differentially expressed genes related to cell cycle were present in GINS4 deficient NK cells. Further, GINS4 variant B cell lines and GINS4-deficient RPE hTERT cell lines had decreased cells entering S phase compared to healthy controls. This indicates a role for GINS4 protein in mitotic exit. Further diverging from DNA damage and DNA damage response phenotype of other helicase mutations,
the GINS4-deficient cell lines displayed only mild increases in DNA damage as evidenced by γH2AX foci, and little or no changes to DNA damage response indicated by ubiquitinated PCNA and pCHK1 compared to healthy controls. GINS4 patient BLCLs also lacked changes in replication fork symmetry or speed as analyzed by DNA fiber analysis, unlike similar experiments in other helicase variants. Although this study lacked an in vivo mouse model, GINS4 contributed to our understanding of the role of the CMG helicase in NK cell development and further highlighted the heterogeneity seen in helicase variants and NKD. It also identified neutropenia to be a common feature resulting from GINS complex variants.

1.11 POLA1

Though not a component of the CMG helicase, DNA polymerase-α (Pol-α) is required for DNA replication initiation. In eukaryotes, the catalytic subunit Pol-α is thought to interact with the helicase through binding to MCM10, triggering replication by generating de novo primers for both the leading and lagging strands (55). Partial hypomorphic Pol-α deficiency has been shown to result in X-linked reticulate pigmented disorder (XLPRD) (87, 127). These patients have overlapping phenotypes with the previously described helicase variant patients, including growth retardation, recurrent infection, hypogonadism, and reduced NK cells with relatively higher frequency of the CD56<sup>bright</sup> subset (87). siRNA against POLA1, the gene encoding for Pol-α, in both primary NK cells and the NK92 cell line led to decreased cytotoxicity against K562 tumor cells (87). Further investigation found that POLA1 and MCM4 expression were linked and together are causative of decreased cytotoxicity of NK cells. However, no DNA damage was indicated in patient cells according to γH2AX or 53BP1 staining, and there was no impairment of proliferation or increase in apoptosis in patient NK cells.
Though the cellular phenotype of DNA damage and proliferative or cell cycle defects was not studied, Pol-α deficiency was linked to a disruption in lytic granule localization to the microtubule organizing center (MTOC) and the immune synapse (IS), which resulted in decreased cytotoxicity (87). This is the first time a helicase deficiency has been linked directly to cytotoxic function, although this phenotype may also reflect a global impairment in NK cell activation leading to a defect in granule convergence during immune synapse formation. Pol-α deficiency further builds on our understanding of how NK cell development and maturation depends critically on DNA replication machinery.

1.12 POLE2

DNA polymerase epsilon (Pol-ε) is involved in synthesis of the leading strand after Pol-α has primed the DNA (128). In addition to the role of leading strand replication, Pol-ε functions in proofreading DNA, regulating cell cycle, and maintaining epigenetics of DNA, and thus is critical for maintaining genome stability (88, 128). Pol-ε closely interacts with several members of the CMG helicase, including the GINS complex and CDC45 during replication (129). The POLE2 gene encodes an important accessory component of pol-ε that is approximately 59 kDa (88, 130). In a brief report by Frugoni et al, an individual was reported to have a homozygous single polymorphic nucleotide (SNP) splice-site mutation at position 1 of intron 13 on POLE2 (88). This individual displayed hypothyroidism and combined immunodeficiency, with T cell lymphopenia, neutropenia, NK cell deficiency with an increased ratio of CD56^{Bright}/CD56^{Dim}, and the absence of circulating B cells, among other immune issues (88). Recurrent respiratory infections were present in this individual as well as systemic BCG infection after vaccination. Though NK cell specific investigation was not conducted, the authors found that T cell stimulation of peripheral blood mononuclear cells (PBMCs) with anti-CD3 with or without anti-CD28 antibodies lead to an
increase proportion of cells undergoing apoptosis by Annexin V staining (88). Further work on this individual’s PBMCs and fibroblasts found a decreased percentage of cells found in S phase, indicating that cell cycle progression is impacted (88). The cell cycle phenotype of patient fibroblasts was partially rescued using wild-type POLE2 lentivirus transfection, resulting in an increased proportion of cells in S phase, despite the percentage of cells in G2M phase remaining high (88). Despite the absence of detailed examination in the NK cell compartment in this individual, the role that various polymerases play in human disease is crucial to our understanding of replication and proliferation in lymphocytes development and function.

1.13 CDC45

Cell division cycle protein 45 (CDC45) is a critical component of the CMG helicase, acting as a rate-limiting factor in DNA replication in mammalian cells (60, 61). As part of the helicase complex, CDC45 is responsible for initiation of DNA replication (56) and DNA double helix unwinding (131). CDC45 deficiency results in Meier-Gorlin syndrome (MGS) (132), a disorder characterized by short stature, small ears, and no or small patellae without immune involvement (133, 134). Here, we will describe a novel CDC45 variant that results in a variable immunodeficiency primarily affecting NK cells, though further investigation is required to connect CDC45 with a molecular mechanism resulting in disease.

1.14 Other genes

Other genes involved in the CMG helicase have been reported to result in human disease. MCM2 deficiency, through autosomal dominant inheritance, results in deafness through selective apoptosis of cells in the ear (135), while MCM3 and MCM5 deficiencies, through autosomal recessive inheritance, can cause Meier-Gorlin syndrome (133, 134). Primary fibroblasts from one MCM5 deficient individual displayed a dysregulation of progression through S phase using a BrdU
pulse-chase experiment. The addition of hydroxyurea to induce replication stress resulted in patient cells remaining arrested in the cell cycle, possibly due to impairment of dormant origins. More recently, both GINS2 and GINS3 deficiency were also described to result in Meier-Gorlin syndrome with autosomal recessive inheritance patterns (136, 137). Though the GINS2 case study did not investigate the stability or expression of other GINS proteins, GINS3 deficiency resulting in MGS also had decreased GINS1 protein expression. Modeling the GINS2 patient variant in yeast cells did not reveal a growth or cell cycle defect when exposed to hydroxyurea, but replication stress was strongly induced in the presence of histone deacetylase inhibitor NAM (137). It should be noted that none of the genes in this section resulted in an immunodeficiency phenotype, despite displaying similar molecular mechanisms to the genes underlying NKD. However, in patients with severe syndromic disease, relatively milder immune deficiencies may not be noted and an in-depth study of the immune profiles of patients with Meier-Gorlin syndrome has not been reported. Finally, a single case study of a homozygous mutation in RTEL1 identified NKD with fatal VZV infection, decreased NK cells in peripheral blood and decreased function without impact on other lymphocyte populations (138, 139). RTEL1, regulator of telomere elongation helicase 1, functions similarly to the CMG helicase in that DNA is stabilized and unwound during telomere elongation. This is supportive evidence natural killer cells are sensitive to a requirement for and reliant upon different types of helicases for maturation and function.

1.15 Abnormal NK cell differentiation in RAG deficient T- B- NK+ SCID and related disorders

Finally, interesting insight can be gained from the study of hypomorphic RAG variants leading to T− B− NK+ severe combined immune deficiency (SCID). Dysfunction of V(D)J rearrangement genes (RAG1, RAG2, DCLRE1C, NHEJ1, and LIG4) causes a broad spectrum of clinical
phenotypes dependent on recombinase activities, including T− B- SCID, Omenn syndrome (OS), atypical SCID (AS), and combined immune deficiency with granuloma and/or autoimmunity (CID-G/A) (140-143). Mutations in these genes can also affect NK cell differentiation and maturation despite the lack of RAG-mediated antigen receptor rearrangement in NK cells, and NK cells from these individuals often have immature phenotypes compared to age-matched healthy subjects (144). Specifically, the frequency of CD56bright NK cells is higher and CD56dim CD16+ are lower in SCID and CID (144). Studies in mice using RAG reporter and fate-mapping systems have demonstrated that RAG expression generates heterogeneity of NK cell origin as some NK cells, but not all, are derived from Rag1+ early progenitors (145). In vitro differentiation of human NK cells from RAG1:GFP iPSC reporter lines also showed that early RAG1+ cells give rise to NK cells, myeloid, and erythroid cells in addition to T/B lymphoid cells (146). While RAG expression could simply be a marker of lymphoid potential, recombinase activity of RAG is not limited to V(D)J recombination during T and B lymphocyte development but also regulates DNA damage recovery and fitness in NK cells (147). RAG-deficient mice have normal NK cell numbers but intrinsic hypo-responsiveness and impaired expansion and survival of NK cells in response to CMV infection (147). Taken together, these data suggest that, while not directly linked to helicase or DNA damage function, activation of DNA damage repair pathways through expression of RAG can confer NK cell fitness and functionality and further link the NK cell phenotype in hypomorphic RAG patients with impaired activation of these pathways.

1.16 Summary

The principal activity of the CMG helicase is facilitating DNA replication while maintaining genomic integrity. Partial loss of function of helicase proteins, including MCM4, GINS1,
MCM10, and GINS4, results in replication stress and DNA damage, ultimately leading to NKD. Helicase proteins clearly play a critical role in human natural killer cells; thus, understanding the role of the CMG helicase through the lens of NKD contributes to our knowledge of NK cell development and function and could potentially unlock novel therapeutic routes for targeting immunodeficiencies.

Chapter 2: Replication stress in activated human NK cells induces sensitivity to apoptosis

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Key words: replication stress, NK cells, cytotoxicity, DNA damage, CMG helicase

Author contributions: E.M.M. conceived the study and conducted microscopy experiments and analyzed images. D.E. supervised research. S.S. prepared samples for and ran western blots. Y-O A. prepared samples for western blots and generated data for pro- and anti-apoptotic proteins using flow cytometry. N.W. completed DNA fiber analysis experiments. R.K.S. performed and analyzed proteomics data. L.A.P. ran chromium release experiments. H.F. conducted microscopy experiments and analysis. N.C.G. performed all other experiments, analyzed data, and wrote the manuscript. All authors reviewed and approved the manuscript.

Introduction

Natural killer cells are innate lymphocytes that lyse virally infected or malignant cells without prior sensitization through germline activating and inhibitory receptors. Human peripheral blood NK cells are present in two subsets; mature CD56\textsuperscript{dim} cells, which make up the majority of circulating NK cells and have greater baseline cytotoxic function, and less mature CD56\textsuperscript{bright} subset, which are potent cytokine producers (4, 6). Despite their lack of germline-encoded antigen specific receptors, NK cells can also have features of immune memory, including rapid expansion upon secondary infection, and epigenetic and transcriptional changes.
that lead to the generation of long-lived memory cells (32-35). Human cytomegalovirus (HCMV) infection can induce the production of memory NK cells through NKG2C-HLA-E interactions (32, 36-38). Other viruses, such as EBV and HIV, induce a subset of long-lived memory NK cells with increased effector functions upon recall (32). Adaptive NK cells can be modeled in vitro using cytokines, termed cytokine induced memory-like (CIML) NK cells, which occurs following stimulation with IL-15, IL-18, and IL-12 and results in NK cell activation of mouse and primary human NK cells (39-41). IL-15 is critical for NK cell maturation and survival, while IL-18 and IL-12 are produced by antigen presenting cells (APCs) in response to viral infection (47, 48). Thus, CIML NK cells proliferate and upregulate cytotoxic responses upon stimulation and can serve as a useful model of primary NK cell activation during viral infection. In vivo, these activated NK cells exhibit heightened responses against infections and cancerous target cells (40, 49, 50).

The absence of NK cells, dysregulation of NK cell maturation, or impairment of NK cell function leads to natural killer cell deficiency (NKD), an inborn error of immunity (IEI) that is associated with recurrent viral infections or cancer (3, 24). Though rare, partial loss-of-function variants in MCM4, GINS1, MCM10, and GINS4 lead to immunodeficiency marked by severe and recurrent viral infections and impaired NK cell maturation and function (26-30). Partial loss-of-function in these contexts is generally defined by decreased protein expression or function while residual activity is maintained. Indeed, total knockout of helicase genes leads to embryonic lethality in multiple mouse models (76, 89, 91). These genes encode components of the CDC45-MCM-GINS (CMG) helicase complex, which is required for DNA replication in S phase by unwinding DNA and recruiting polymerases (53, 54). The CMG helicase is made up of a core of 11 subunits including CDC45, MCM2-7 and GINS1-4 with additional proteins including
The helicase is active in any proliferative cell and plays an important role in maintaining genomic stability (53, 54). Individuals with NKD due to partial loss-of-function of helicase proteins have increased DNA damage and apoptosis, cell cycle arrest, and replication stress in NK cells and other cell subsets, however the mechanism behind the accumulating evidence that NK cells are more sensitive than other lymphocytes or proliferating cells is poorly understood (26-30, 148).

Replication stress occurs when a replication fork is stalled or traveling too slow for proper function (149). Stress at the replication fork arises from endogenous sources including DNA lesions such as inter-strand crosslinks (ICLs), dormant replication origins, and erroneous incorporation of nucleotides during replication in S phase (149). Dysregulation of CMG helicase proteins leads to replication stress. Overexpression of Cdc45 in *Xenopus* extracts results in replication stress through increased origin firing and density, and increased asymmetrical replication forks (150). The same study overexpressed the GINS complex and found similar results with decreased inter-origin distance and similarly increased fork asymmetry (150).

Transient knockdown of *MCM3* and *MCM2* with siRNA similarly resulted in replication stress as evidenced by DNA damage and activation of the DNA damage response (DDR) pathway (151). RNAi against MCM5 developed increased sensitivity to replication stress from chemical inhibition of replication with an accumulation of DNA damage and cell cycle arrest phenotypes (152). The CMG helicase complex and associated proteins also serve as molecular protectors against endogenous causes of replication stress. MCM10, which activates origins and maintains stability during elongation, can balance replication stress in cancer cells by activating dormant origins (31, 153, 154). Thus, loss of helicase function results in replication stress. However, NK cells are particularly susceptible to helicase protein deficiency while other lymphocytes such as
CD3+ T cells appear to exhibit tolerance. Helicase deficiency may have an impact on the development of NK cells, such as blocking the maturation into the CD56\textsuperscript{dim} subset (26-29). However, while helicase deficiencies have been shown to affect NK cell development, the ubiquitous expression of helicase proteins led us to predict that partial loss-of-function variants can also affect mature NK cell function. We thus sought to investigate whether the unusual susceptibility of NK cells to replication stress induced by helicase deficiencies affects mature NK cell function in addition to NK cell maturation.

Here, we demonstrate that mature NK cell subsets are more sensitive to replication stress than T cells during activation. We find that, as previously shown, the CD56\textsuperscript{bright} NK cell subset is the most proliferative subset during activation with CIML stimulation and is also more sensitive to replication stress than CD56\textsuperscript{dim} NK cells. CD56\textsuperscript{bright} cells undergo caspase mediated apoptosis in response to replication stress induced by knockdown of MCM10 or treatment with a low dose of the replication inhibitor aphidicolin. The survival of T cells in response to CD3/CD28/IL-2 activation and replication stress remains relatively unaffected despite undergoing similar changes in replication fork speeds in response to aphidicolin treatment. We additionally found that cytotoxic protein expression decreases with increasing replication stress, which provides a link between replicative stress and NK cell function. Finally, cell fate decisions due to DNA damage and replication stress are maintained in part by a balance of apoptosis and autophagy. We found that the CD56\textsuperscript{dim} subset undergoes autophagy in response to activation, though replication stress during activation does not further increase autophagy. Together, these results reveal insight into the mechanisms of NK cell deficiency produced by helicase deficiencies and replication stress and uncover novel aspects of NK cell biology and function.
2.1 NK cell activation induces cell cycle dependent DNA damage

To better understand the effect of helicase deficiency on mature NK cells, we first sought to investigate proliferation and DNA damage in the context of activation. Activated NK cells were generated by overnight priming of healthy donor PBMCs with IL-15, IL-12, and IL-18 (CIML) followed by low dose IL-2 for one to four days (Fig. 2.1A) (40, 41, 50). Controls were given only low dose IL-2 or IL-15 for the same amount of time. Using BrdU, we found that CIML NK cells have a significantly increased frequency of cells found in S phase four days after stimulation relative to incubation with IL-15 only or no prime control (low dose IL-2 only) (Fig. 2.1B). Consistent with increased progression to S phase, we also confirmed that cell proliferation, a positive indicator for cellular activation (155), was increased in this CIML population when measured by CF-SE (Fig. 2.1C).

To determine the effect of NK cell activation on conventional markers of DNA damage, we measured γH2AX by flow cytometry following activation by CIML cytokines or IL-15 alone normalized to the no prime control. CIML stimulated NK cells had significantly more γH2AX positive cells compared to IL-15 alone (Fig. 2.1D). To determine whether induction of DNA damage observed in cytokine primed NK cells is a conserved response amongst lymphocytes after activation, we compared activated NK cells with CD3+ T cells stimulated with anti-CD3/-CD28 magnetic beads and IL-2 (T stim). We also tested CIML conditions on T cells as an additional control to validate our T cell specific stimulation. CD4+ and CD8+ T cells consistently increased expression of activation markers CD69 and CD25 (156, 157) after three days of continuous T cell specific stimulation, whereas CIML cytokines did not have the same effect (Supp Fig 2.1A). We additionally confirmed using CF-SE staining that the proliferation of activated T cells was increased compared to no stimulation controls or CIML stimulation (Fig.
2.1E). Despite their robust activation and proliferation, T cells activated with T cell specific stimulation had only moderate increases in DNA damage measured by γH2AX when normalized to an unstimulated control or compared to mild activation with CIML cytokines (Fig. 2.1F). In summary, stimulation of NK and T cells robustly induces proliferation in both populations to a similar extent when measured by CF-SE, however activation leads to a greater frequency of NK cells with DNA damage.
Figure 2.1

A

PBMCs

No stimulation (T), No prime: continuous low dose IL-2 (NK)

Continuous low dose IL-15

PBMCs

Overnight Prime:
IL-15, IL-18, IL-12 (CIML)

/+ aphidicolin 1 to 3 days

Low dose IL-2

PBMCs

/+ aphidicolin 1 to 3 days

Expanded NK cells

Expanded T cells

B

% NK cells in S phase

No Prime  |  CIML  |  IL-15

C

% Proliferated NK cells

No Prime  |  CIML  |  IL-15

D

% Proliferated NK cells

CIML  |  IL-15

γH2AX

No Prime  |  CIML / IL-15
Aphidicolin treatment impairs replication fork progression in NK and T cells

We sought to use mild replication stress to model the effect of helicase deficiencies on NK cells and T cells following their activation. At low doses such as 0.25 μM and 0.5 μM, aphidicolin induces replication stress, while higher doses like 1 μM and 5 μM result in cell cycle arrest (158, 159).
Following CIML cytokine priming overnight, NK cells were cultured with low dose IL-2 in the presence or absence of 0.25 µM aphidicolin for 72 hours. NK cell survival in the absence of activation was tested using the no prime condition but continuous low dose IL-2. Induction of replication stress with aphidicolin leads to a similar phenotype as previously reported in cells from helicase-deficient patients, particularly the MCM10-deficient individual (27), including DNA damage and cell cycle arrest in early S phase (Supp. Fig. 2.3A). In parallel, we performed T cell stimulation as previously described and including a continuous low dose IL-2 control, followed by aphidicolin treatment for the same length of time as NK cells.

Activation of NK cells without aphidicolin did not induce cell death as detected by propidium iodide (PI) staining. However, low dose replication stress during activation significantly increased the frequency of PI+ NK cells (Fig. 2.2A). In contrast, while T cell activation increased the frequency of PI+ T cells relative to the low dose IL-2 control, there was no significant increase in this population with 0.25 µM aphidicolin (Fig. 2.2A). We sought to determine whether the differential response we observed between activated T cells and NK cells was due to differences in the effect of aphidicolin on replication fork speed. DNA fiber assays were performed on activated T cells or NK cells in the presence or absence of 0.25 µM aphidicolin. Replication fork speed in the absence of aphidicolin was not significantly different between NK cells and T cells. Notably, two hours of aphidicolin treatment in the presence of cell type specific activation similarly decreased fork speeds in both NK and T cells (Fig. 2.2B), suggesting that the differences in response to 0.25 µM aphidicolin were not due to baseline differences in fork speed between NK cells and T cells or to differential effects of aphidicolin on DNA replication fork progression.
We predicted that the addition of aphidicolin would further increase the presence of γH2AX induced by activation of NK cells. As CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells have differential proliferative responses to CIML activation (160), we measured these subsets, and CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell subsets, independently following cell type specific activation or low dose IL-2. γH2AX intensity was significantly increased in the CD56\textsuperscript{bright} subset in response to aphidicolin (Fig. 2.2C). The CD56\textsuperscript{dim} population had increased DNA damage in response to activation but did not further accumulate damage with the addition of replication stress (Fig. 2.2C). Similarly, both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell populations accumulated low amounts of DNA damage with stimulation and maintained this low level even with replication stress (Fig. 2.2C). These data demonstrate that CD56\textsuperscript{bright} NK cells are specifically sensitive to replication stress.

We sought to measure additional indicators of replication stress and DNA damage. Exposed single stranded DNA (ssDNA) is present during homology directed repair and in the process of proliferation and replication activities (161). The replication protein A (RPA) binding stabilizes ssDNA and facilitates downstream DNA damage repair activities (162). To measure the presence of ssDNA, we performed flow cytometry BrdU assays in the absence of the commonly used DNA denaturing step. In CD56\textsuperscript{bright} NK cells, we found an increase in exposed ssDNA in response to activation (Fig. 2.2D, E). The addition of low dose aphidicolin decreased the percentage of BrdU positive cells, likely due to decreased cell cycle progression (Fig. 2.2D, E). CD56\textsuperscript{dim} cells also increased ssDNA in response to activation, though not to the same degree as the CD56\textsuperscript{bright} subset. Both subsets of T cells maintained low levels of ssDNA that were seemingly independent of activation or replication stress (Fig. 2.2D, E). We found a trend similar to γH2AX distribution when we analyzed phosphorylated RPA (phospho-RPA Thr21) by flow cytometry and found that the CD56\textsuperscript{bright} NK cell subset had increased phospho-RPA (Thr21).
following treatment with 0.25 μM aphidicolin (Fig. 2.2F). In contrast, the CD56dim subset and T cell subsets maintained low levels of phospho-RPA regardless of activation or replication stress (Fig 2.2F). Confocal microscopy showed a greater number of γH2AX and phospho-RPA (Thr21) foci in activated NK cells than T cells (Fig. 2.2G) and this observation was supported by quantification (Fig. 2.2H). We further tested the drug etoposide, which is inhibits synthesis phase by binding to topoisomerase II and DNA complexes (163), and displayed similar effects on CD56bright and CD56dim in terms of NK cell survival and DNA damage accumulation (Supp Fig 2.2A-C). These data indicate that the CD56bright subset is affected by mild replication stress while undergoing CIML activation to a greater extent than CD56dim NK cells or T cell subsets being activated by T cell specific stimulation. They further show that, despite equivalent replication fork speeds and response to aphidicolin, NK cells and T cells respond differently to mild replication stress.

![Figure 2.2](image-url)
Figure 2.2. Aphidicolin treatment impairs replication fork progression in NK cells and T cells. A) Percent propidium iodide (PI) positive CD56<sup>+</sup>CD3<sup>+</sup> NK cells and CD3<sup>+</sup> T cells following low dose IL-2 or NK (CIML) or T cell activation with or without aphidicolin treatment. (B) Fork speed in kilobases per minute of enriched stimulated NK cells or T cells from a DNA fiber analysis assay. Cells were incubated for two hours with 0.25 µM aphidicolin following overnight cytokine stimulation for enriched NK cells or continuous anti-CD3 -CD28 beads with 30 U/mL IL-2 for T cells. Cells from two healthy donors were pooled to make the DNA fiber slides. (C) Percentage of γH2AX<sup>+</sup> cells in NK and T cell subsets with or without low dose aphidicolin following activation with low dose IL-2 or CIML activation (NK cells) or T cell activation. (D) Percentage of BrdU<sup>+</sup> cells without DNase treatment (exposed ssDNA) measured by flow cytometry. (E) Example flow plots are shown with BrdU<sup>+</sup> gating strategy based on BrdU FMO control with DNA content on the X-axis. (F) Phospho-RPA (Thr21)<sup>+</sup> cells in NK and T cell subsets with or without low dose aphidicolin following activation with low dose IL-2 or CIML activation (NK cells) or T cell activation. Cells are gated based on fluorescence minus one or no stain controls. (G) Representative confocal microscopy images from CIML activated NK cells (top) and activated T cells (bottom). (H) Quantification of γH2AX or pRPA (Thr21) foci in CIML NK cells and activated T cells. N=4 biological replicates. Data are shown as mean ± SEM. Two-way ANOVA comparing cell subsets was performed for multiple comparisons.
2.3 Replication stress leads to apoptosis of activated NK cells

Next, we sought to further investigate the mechanisms of increased NK cell death in response to activation and replication stress. The tumor suppressor transcription factor p53 plays a role in controlling cell fate, including cell cycle arrest, repair, senescence, or apoptosis (164). We investigated phospho-P53 Serine 15 (pP53) and p21, which is a downstream target of pP53, by flow cytometry for NK and T cell subsets. As shown in Fig. 2.3A, the CD56\textsuperscript{bright} NK cells had highest expression of pP53 following activation, with or without replication stress. The CD56\textsuperscript{dim} NK cell subset had moderate levels of pP53 that were increased relative to no prime controls. However, T cells maintained low levels of pP53 and these were independent of aphidicolin treatment, and neither T cells nor NK cells had significant pP53 expression in response to low dose IL-2 controls. Analysis of mean fluorescent intensity (MFI) displayed a similar trend, where activated CD56\textsuperscript{bright} cells have increased p21 compared to no prime controls, without further impacts by replication stress (Supp. Fig 2.3A). p21 MFI in CD56\textsuperscript{dim} NK cells and CD3\textsuperscript{+} T cells was unchanged by activation or replication stress (Supp. Fig 2.4A). Next, we measured pan-caspase expression by flow cytometry to determine signaling pathways resulting in apoptosis. NK cells had greater caspase activation with increasing replication stress, whereas T cells maintained a low level of caspase activity even in the presence of aphidicolin and both cell types had low caspase expression in low dose IL-2 conditions (Fig. 2.3B). This result was further confirmed by MitoNIR staining, which decreases in intensity with loss of mitochondrial membrane potential, indicating cell death (165). Here, we found moderate increases in the percentage of cells that were MitoNIR\textsuperscript{low} with replication stress for both CD56\textsuperscript{bright} and CD56\textsuperscript{dim} populations, with both populations having higher frequencies of MitoNIR\textsuperscript{low} cells than either T
cell subset (Fig. 2.3C). CD4+ and CD8+ T cells did not have increased MitoNIRlow populations following aphidicolin treatment.

We also conducted proteomics analysis of activated NK and T cells and found variable differences in pro-apoptotic proteins levels (Fig. 2.3D). Notably, NK cells upregulated CASP7 and CASP3 compared to T cells, however, several proteins were not detected in activated NK cells, such as TOP2A, XIAP, FADD, and DFFA (Fig. 2.3D). To complement the proteomics analysis, we investigated anti-and pro-apoptotic proteins by flow cytometry in activated NK cells with and without replication stress, including Bcl-XL (Fig. 2.3E) and BIM (Fig. 2.3F), respectively. Here, we find that both subsets of NK cells had increased protein expression with CIML activation relative to low dose IL-2 only, while the addition of aphidicolin to induce replication stress did not further increase protein expression. Thus, NK cell activation, and not just the addition of replication stress, induces the upregulation of both anti- and pro-apoptotic factors, indicating a change in the balanced expression of apoptotic proteins.

Finally, we asked if the process of autophagy could be playing a role in lymphocyte response to replication stress. Autophagy is an important recycling process necessary for maintaining cellular homeostasis (166). Autophagy may be activated by the same stresses as apoptosis, such as in response to replication stress and DNA damage (166, 167). Here, we used the fluorescent probe Autophagy Red, which is detected in the lipid membrane of autophagosomes and autolysosomes. We found that the CD56dim subset had the highest percentage of cells undergoing autophagy following activation in the presence or absence of replication stress (Fig. 2.3G). Relative to CD56dim NK cells, CD56bright NK, CD4+ and CD8+ T cells maintained low levels of autophagy following activation with or without replication stress (Fig 2.3G). Through pP53 signaling, loss of mitochondrial membrane potential, and caspase
activation, apoptosis occurs in CD56\textsuperscript{bright} NK cells in response to activation. Caspase activity was particularly sensitive to replication stress in NK cells. The CD56\textsuperscript{dim} subset of NK cells undergoes autophagy in addition to apoptosis in response to activation and replication stress. Together, these data indicate that NK cells, especially the CD56\textsuperscript{bright} subset, exhibit differential sensitivity to replication stress compared to T cells.
2.4 Differential expression of CMG complex members in NK and T cells

The function of CMG helicase proteins is tightly linked to their expression (150-152), and loss-of-function variants in MCM4, GINS1, MCM10 and GINS4 lead to immune deficiency. We hypothesized that differences in the cell-intrinsic expression of CMG helicase complex proteins could account for the differential response to replication stress between NK cells and T cells. We performed Western blotting on isolated resting NK cells and T cells from three healthy donors. GINS4 and CDC45 were selected as representative proteins within the CMG helicase. We found no difference in the relative expression of GINS4 (Fig. 2.4A) and CDC45 (Fig. 2.4B) in resting NK and T cells from three donors when normalized. Next, we activated NK cells with high and low doses of IL-15 to induce activation with less cell death than CIML conditions (10 ng/mL and 1 ng/mL, respectively) and T cells with soluble anti-CD3 and -CD28 with soluble IL-2. Activation led to increased expression of GINS4 with a greater increase in expression in T cells than NK cells (Fig. 2.4C). In contrast, we did not detect a consistently significant increase in CDC45 protein expression between conditions due to variability in expression between donors (Fig. 2.4D). To perform further unbiased comparison of changes in protein expression, activated NK and T cells from three healthy donors were evaluated by proteomics analysis after CIML or T cell activation, respectively. Here, we consistently found significantly increased MCM proteins...
in activated T cells compared to NK cells (Fig. 2.4E, F, G). Similarly, we found increased levels of other canonical proteins required for replication, including PCNA and POLD1, in T cells but not NK cells (Fig. 2.4H, I). We included ACTB as a housekeeping protein control, which did not identify significant differences between activated NK and T cells (Fig 2.4J).

Next, we analyzed the primary cell RNA-seq atlas data from BioGPS (168), as it allowed us to compare helicase and DNA replication factors expressions in resting versus IL-2 activated NK cells and naïve versus stimulated T cells. We found a consistent pattern of increased gene expression in response to T or NK cell activation in GINS4 (Fig. 2.4K), CDC45 (Fig. 2.4L), MCM10 (Fig. 2.4M), and POLD1 (Fig. 2.4N). ACTB, included as a control protein, shows similar increases for stimulated NK and T cells (Fig 2.4O). Notably, stimulated T cells tended to have increased gene expression in response to activation to a greater extent than NK cells, a trend that was consistent for other helicase-related genes including GINS1, GINS3, MCM2, MCM6, MCM7, and POLA1 (Supp. Fig. 2.5A-F). Thus, differential gene expression changes, namely increased expression in response to activation, could contribute to the greater ability of T cells to withstand mild replication stress relative to NK cells.
2.5 Replication stress impairs the cytotoxic capacity of primary human NK cells

Replication stress induced apoptosis and autophagy in CD56\textsuperscript{bright} and CD56\textsuperscript{dim} cells respectively (Fig. 2.3), though some cells do survive after presumably repairing DNA damage and restarting the cell cycle. We investigated the cytotoxic capacity of NK cells that had survived replication stress by measuring lytic effector molecules and directly testing cytolytic function of primary NK cells against target cells. We found that activation in the presence of aphidicolin led to a decreased frequency of granzyme B positive CD56\textsuperscript{dim} and CD56\textsuperscript{bright} cells in a dose-dependent manner (Fig. 2.5A). CD56\textsuperscript{dim} cells followed this trend for granzyme A, which triggers cell death through mitochondrial breakdown (169), though the mean fluorescence intensity (MFI) remains the same or increases. However, the CD56\textsuperscript{bright} subset were less affected by replication stress when considering the percentage of granzyme A positive cells (Fig. 2.5B). The percent of perforin positive CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cells also decreased with increasing replication stress (Fig. 2.5C). Interestingly, IFN-\(\gamma\) production of CD56\textsuperscript{bright} NK cells was unaffected by replication stress and even 5 \(\mu\)M aphidicolin did not affect IFN-\(\gamma\) production by surviving cells, despite CD56\textsuperscript{bright} NK cells being the subset most strongly affected by replication stress (Fig. 2.5D). Finally, we directly tested the lytic capacity of replication stress-affected NK cells on K562 leukemic target cells. Using healthy donor activated and enriched NK cells from PBMCs,
we found decreased percent specific lysis against target cells, though no statistical significance was determined (Fig. 2.5E). Thus, in addition to the replication stress in activated NK cells resulting in apoptosis and autophagy, the surviving cells are also mildly affected showing decreased expression of effector proteins that can lead to decreased cytotoxicity against target cells.

![Figure 2.5](image)
Helicase protein knockdown leads to increased NK cell apoptosis

Previously, our lab tested the effect of MCM10 knockdown in the NK92 NK cell line using CRISPR-Cas9 (27). MCM10-knockdown (KD) NK92 cells exhibited increased frequency of cells in early S phase and increased doubling time, indicative of replication stress (27). Induction of replication stress with aphidicolin leads to a similar phenotype as previously reported in cells from helicase-deficient patients, particularly the MCM10-deficient individual(27), including DNA damage and cell cycle arrest in early S phase (Supp. Fig. 2.3A). To further model the MCM10 loss-of-function variants in an NK cell line with a terminally mature NK cell phenotype (170), we targeted MCM10 expression using shRNA in the YTS human NK cell line. Western blot confirmed decreased protein expression by about 80% in the MCM10 knockdown (MCM10-KD) cell line compared to scramble shRNA control (Fig. 2.6A). We also found a higher proportion of knockdown cells were PI positive by flow cytometry, indicating poor cell survival of MCM10-KD cells (Fig. 2.6B). BrdU incorporation to investigate cell cycle revealed a significantly higher percentage of MCM10-KD YTS cells in S phase, specifically early S phase (Fig. 2.6C). Taken together, these data recapitulate previously described impairment in cell cycle
progression with a greater number of cells in early S phase resulting from loss of MCM10 function (27) and further revealed impaired cell survival resulting from MCM10-knockdown.

Next, we sought to quantify the effect of MCM10 knockdown on markers of replication stress. We performed confocal imaging of nuclear area, phospho-RPA (Thr21) foci, and γH2AX foci in MCM10-KD and scramble control lines. Image analysis revealed increased nuclear area (Fig 2.6D), phospho-RPA foci, and γH2AX foci (Fig. 2.6E). This is in line with the MCM10-deficient patient where increased nuclear area and γH2AX foci were seen (27). We also measured apoptosis in the MCM-KD and control cells and found the percentage of pan-caspase positive cells was increased in MCM10-KD (Fig. 2.6F). Given the impaired cytotoxic function of primary NK cells activated and treated with aphidicolin, we sought to determine the effect of MCM10-KD on YTS cell function. There was a consistent decrease in intracellular granzyme B (Fig. 2.6G) and perforin (Fig. 2.6H) relative to scramble shRNA controls, however this decrease was not statistically significant (Fig. 2.6G, H). Together, these data indicate that decreased expression of helicase proteins can recapitulate the phenotype described earlier in replication stress-induced activated healthy donor NK cells. Thus, helicase deficiency as a result of loss of function or decreased expression of core replisome proteins results in replication stress, which is ultimately the driver of apoptosis and decreased cytotoxicity in NK cells but not in activated T cells.
Figure 2.6

A. Relative Expression (A.U.)
- MCM10
- Actin

B. % PI+ cells
- Normalized to Mode

C. Cell incorporated BrdU
- S phase: 30.2%
- S phase: 41.2%

D. Nuclear Area (μm)

E. # pRPA Foci per cell

F. % Caspase+ cells

G. Relative Fluorescence Intensity

H. Relative Fluorescence Intensity

PI

Granzyme B

Perforin
2.7 Discussion

The association between partial loss-of-function variants in genes encoding the CMG helicase and NKD have been well documented (26-30). Cellular phenotypes of these variants include cell cycle arrest, DNA damage, and replication stress (26-30). While these variants impair NK cell development, they also negatively affect the proliferative response of mature NK cell subsets (26, 27, 29, 30), while T cell responses are often intact or less affected than NK cells (26, 27, 29). Here, we use DNA polymerase inhibition with aphidicolin to increase replication stress in primary NK cells and gene knockdown to recapitulate the effects of helicase deficiency in an NK cell line. Aphidicolin was used throughout our study as a means to delay replication by inhibiting DNA polymerase. Our lab and other have shown that limitations to helicase function through helicase deficiency leads to DNA damage, replication stress and cell cycle arrest, which is a phenotype that also appears when aphidicolin is used at low doses. We show that replication stress induced by aphidicolin strongly impacts the CD56bright NK cell subset by increasing γH2AX and phosphorylated RPA and ultimately triggers an apoptotic program in NK cells but not T cells. These data provide mechanistic insight into the nature of immunodeficiency in individuals with helicase variants. They also provide interesting new biological insight into how
NK cells and T cells differ in response to replication stress as well as helicase protein expression, despite both cell types being cytolytic effector cells.

We explored helicase protein expression in resting and activated NK and T cells and found that T cells upregulated helicase genes and proteins to a higher degree than NK cells. Increased CMG helicase protein expression has been associated with increased proliferation, especially in relation to cancer and reviewed in full elsewhere (51, 171-175). Here, we show that activated NK cells and T cells proliferate at a similar rate and have similar replication fork speeds in our in vitro assays, yet T cells have a greater increase in helicase protein expression in response to activation. Perhaps T cell control of helicase proteins during proliferation offers protection against DNA damage and replication stress by allowing multiple dormant origins to fire at once and maintaining adequate DNA damage response signaling (63). The MCM paradox states that excess inactive MCM proteins are loaded onto chromatin, yet only a fraction of these are licensed into active replication forks during S phase (62). A leading theory towards understanding this paradox hypothesizes that the excess MCM proteins are necessary to trigger dormant origins during replication stress (62). Thus, T cell upregulation of helicase proteins during activation could combat replication stress by initiating dormant replication origins. Conversely, NK cells could use a lower threshold of helicase proteins during proliferation but are left at risk of programmed cell death in response to stimulatory signals.

It is pertinent to note that activated NK cells have significant DNA damage when compared to unstimulated controls, indicating that NK cells during viral infection will likely accumulate DNA damage just by being activated and could undergo activation-induced cell death (AICD). In support of this, Terren et al demonstrated that CIML activation of NK cells resulted in decreased viability, likely due in part to failed mitochondrial dynamics and
mitophagy. Apoptosis resulting from activation was further confirmed with accumulation of superoxide and release of cytochrome c in NK cells (176). T cells, particularly CD8+ T cells, undergo AICD as a form of peripheral immune regulation with signaling through Fas/FasL or TNFR2, which is particularly important for the prevention of autoimmune disease (177, 178). However, a threshold for AICD in NK cells after activation may be contributing to our results where we find a higher sensitivity to DNA damage and replication stress. Research to clearly define AICD in NK cells would further this hypothesis and improve our biological understanding of NK cells responses during human disease.

Understanding natural killer cell requirements for replication, especially under conditions of stress, is paramount for deciphering the role of the CMG helicase in these cells. Our work and previous helicase NKD studies have determined that NK cell maturation is blocked, particularly through apoptosis of the CD56\textsuperscript{bright} population. Gineau et al found that CD56\textsuperscript{bright} NK cells from MCM4 deficient individuals had chromosomal aberrations, indicative of replication stress, and had increased apoptosis (30). In our data, aphidicolin-induced replication stress resulted in apoptosis of the CD56\textsuperscript{bright} population, which left behind a small population of cells that can survive replication stress yet have impaired cytotoxicity. While this study was limited to CIML activation of NK cells, it addresses fundamental differences between NK and T cells that contribute to human disease. It also provides the first mechanistic understanding of previously observed susceptibilities of NK cells to mild replication stress. Considering our results here, we have identified a connection between helicase deficiency and replication stress leading to NK cell-specific dysfunction. Our work shows that human NK cells and T cells have differential sensitivities to replication stress, and additional research should further study the connection between helicase proteins and disease. Better understanding of how cytokine-induced activation
affects NK cell survival, and the effect of mild replication stress on activated NK cells, can help better design strategies for the use of immunotherapeutic NK cells.
Chapter 3: CDC45 haploinsufficiency as a novel cause of natural killer cell inborn error of immunity (NK IEI)

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Key words: NK cells, cytotoxicity, CMG helicase, CDC45, Inborn Errors of Immunity

Author contributions: E.M.M. conceived the study. M.I.C. created BLCLs and all associated experiments and prepared samples for and analyzed Nanostring data. S.A.S. conducted experiments on BLCLs. E.H.S. performed flow phenotyping experiments. M.P. ran chromium release assays and phenotyping experiments. A.V.H. conducted phenotyping experiments by flow cytometry. N.L. analyzed Nanostring data. I.K.C. analyzed WES data and J.R.L. was supervising geneticist. N.C.G. performed all other experiments, analyzed data, and wrote the manuscript.
Introduction

Natural killer cell deficiency (NKD) is a primary immunodeficiency where natural killer cells are the primary lineage affected (3, 24). Individuals with NKD present with malignancies and susceptibility to viral infection, especially from the Herpesviridae family. Though NKD is rare, four genes that are involved in the CDC45-MCM-GINS (CMG) helicase have been described to result in NKD, including MCM4 (28, 30), GINS1 (29), MCM10 (27), and GINS4 (26). The CMG helicase is critical for eukaryotic replication and plays a critical role in maintaining genomic stability (54). To our knowledge, CDC45 deficiency has never been described as a monogenic cause of NKD. However, here we describe a single damaging heterozygous variant in CDC45 in an individual with consistent defects in the NK cell compartment, in addition a diagnosis of common variable immunodeficiency (CVID). This individual has a disruption in the ratio of CD56<sup>bright</sup> to CD56<sup>dim</sup> cells, severely decreased frequency of adaptive NK cells, and decreased NK cell cytotoxic function. As previously reported for other helicase variants that cause NKD, we found cell cycle defects, increased DNA damage, and impaired DNA damage repair signaling in patient lymphocytes. The description of CDC45 as a cause of NK IEI contributes to our understanding of the role of the CMG helicase in NK cell maturation, function, and biology.

3.1 Clinical phenotype and immune features

The 56-year-old female proband presented with CVID in adolescence, with near absent IgA and IgM (Table 3.1). The patient has recurrent herpes simplex virus (HSV) that requires anti-viral prophylaxis and exhibits recurrent zoster with femoral neuralgia. Chronic mastoiditis and otitis media are present as well as hypothyroidism and idiopathic osteoporosis. This individual also has suspected lymphocytic meningitis and a family history of testicular cancer in her brother.
Clinical lab data show consistently low CD3⁺CD4⁺ T cells, occasionally low CD20⁺ B cells and variably low NK cell numbers in peripheral blood (Table 3.2). Mitogen responses and antigen stimulation were found to be normal in this patient, with the exception of low responses to tetanus antigen (Table 3.3).

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<td>&lt;6 (L)</td>
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<td>10 (L)</td>
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<td>11 (L)</td>
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**Table 3.1 Immunoglobulin levels**
(L) levels lower than lab range, (H) levels higher than lab range
Cells left empty were not measured at that date
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<td>1056</td>
<td>646</td>
<td>1,176</td>
<td>663</td>
<td>735</td>
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<td>CD3+</td>
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<td>908</td>
<td>549 (L)</td>
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<td>CD3+CD4+</td>
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<td>488 (L)</td>
<td>297 (L)</td>
<td>497 (L)</td>
<td>280 (L)</td>
<td>264 (L)</td>
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<td>CD3+CD8+</td>
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<td>391</td>
<td>239</td>
<td>300</td>
<td>212</td>
<td>260</td>
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<td>CD19+</td>
<td>63 – 461</td>
<td>74</td>
<td>52 (L)</td>
<td>94</td>
<td>46 (L)</td>
<td>37 (L)</td>
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<td>CD3-CD56CD16+</td>
<td>89 – 472</td>
<td>63 (L)</td>
<td>39 (L)</td>
<td>118</td>
<td>80 (L)</td>
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<tr>
<td>CD8+CD56+</td>
<td>30 – 200</td>
<td>106</td>
<td>223 (H)</td>
<td>133</td>
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Table 3.2: Clinical Labs
(L) levels lower than lab range, (H) levels higher than lab range
Cells left empty were not measured at that date

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<tr>
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<th>Proband 2/2017</th>
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<th>Control 2/2017</th>
<th>Control 8/2018</th>
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<td>218,851</td>
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<td>317,835</td>
<td>163,507–415,087</td>
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<td>ConA 50 µg/mL</td>
<td>149,192</td>
<td>290,372</td>
<td>243,015</td>
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<td>PWM 100 ng/mL</td>
<td>113,532</td>
<td>109,241</td>
<td>125,201</td>
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<td></td>
<td>864</td>
<td>771</td>
<td>215 – 1,161</td>
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<td>----------------</td>
<td>-----------</td>
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<tr>
<td>Unstimulated</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Antigen: Candida</td>
<td>15,396 (net)</td>
<td>5,317 (net)</td>
<td>7,025 (net)</td>
<td>85,874 (net)</td>
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<td></td>
<td>10.80 (index)</td>
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<td>5.57 (index)</td>
<td>188.09 (index)</td>
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<td>1,684 (net)</td>
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<td>86,760 (net)</td>
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<td></td>
<td>1.84 (index)</td>
<td>13.60 (index)</td>
<td>190.02 (index)</td>
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<td>Antigen Unstim</td>
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Table 3.3: Mitogen and Antigen Stimulation (L) levels lower than lab range, (H) levels higher than lab range Cells left empty were not measured at that date

3.2 NK cell phenotype and decreased cytotoxic function

Despite the involvement of other immune lineages, including CD20<sup>+</sup> B cells and CD4<sup>+</sup> T cells, NKD was suspected due to consistently low NK cell numbers in peripheral blood, low NK functionality, and recurrent viral infections of the Herpesviridae family, namely HSV and VZV. We performed NK cell phenotyping and functional analysis across several timepoints. We found variable percentage of NK cells in peripheral blood, ranging from 2% to >12% from PBMCs and the representative flow plot shows normal NK cell numbers in peripheral blood (Fig 3.1A). However, we did find consistently imbalanced ratio of CD56<sup>+</sup> to CD56<sup>dim</sup> NK cells and low numbers of adaptive NK cells, indicating a failure of maturation in NK cells (Fig 3.1B and C). We tested the functionality of the proband’s NK cells and found that the % specific lysis against K562 erythroleukemia target cells was consistently decreased compared to healthy donors (Fig 3.1D). Alongside this data, we investigated the cytotoxic granule protein perforin and found
decreased percent of positive NK cells as well as mean fluorescence intensity (Fig 3.1E). We additionally used Nanostring gene expression array, which analyzed 500 immune-related genes, and further confirmed that perforin was decreased in the proband compared to three healthy donors (Fig 3.1F). Lastly, using confocal microscopy to image conjugate pairs of NK cells killing K562 target cells found no significant differences in several metrics of cytotoxicity. There were no differences in distance between perforin granules and the immune synapse (IS) or the microtubule organizing center (MTOC), and no difference in distance between the MTOC and the IS (Figure 3.1G and H). This indicates that the critical steps of immune synapse formation are intact, but target cell killing on a population level is impaired. Thus, NK cell phenotyping supports the conclusion that this individual consistently exhibited a defect in the NK cell compartment, consistent with a diagnosis of NK IEI.

![Figure 3.1](image-url)
A genetic cause for NK IEI was investigated since an immune defect was confirmed within NK cells of this individual. The proband is depicted as the red circle in the pedigree, and the status of the immediate family including an unaffected mother and two brothers are shown (Fig 3.2A). It
should be noted that one brother has the same variant as the proband, yet remains unaffected without signs of NK IEI. This variant was found by whole exome sequencing (WES on DNA from blood samples of the proband and her mother and analyzed with respect to Mendelian inheritance patterns. The variant was found in the \textit{CDC45} gene found on chromosome 22. At hg38 chr22: 19,482,781 in exon 4, two nucleotides (TG) were inserted. This resulted in a frame shift mutation in the protein at phenylalanine at residue 96. The frameshift results in the creation of three novel amino acids with the premature termination codon truncating the remainder of the protein, causing a deletion of the critical nuclear localization sequence and presumably any other binding domains (mutationtaster.org). This result was confirmed in the proband with Sanger sequencing (Fig 3.2B). This variant was not previously documented in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar) and a CADD score could not be generated. The variant was not detected in ExAC database nor 1000G. This variant is found in advance of the nuclear localization sequence (NLS), as depicted in the CDC45 protein schematic (Fig 3.2C) and the truncated protein structure is predicted in 3D projection (Fig 3.2D). An immortalized B cell line was created from healthy donor and patient PBMCs by co-incubating them with EBV and B95-8 Marmoset cells. Using an antibody against the C-terminal domain of CDC45, approximately 50\% of protein was found compared to three healthy donors in B cell line cells (BLCLs) (Fig 3.2E). It should be noted that truncated CDC45 protein could not be detected using an antibody against the N-terminus domain, indicating that nonsense mediated decay is likely responsible for the degradation of the variant allele product.
Figure 3.2

A

WT/WT

WT/F96fs

WT/F96fs

B

Mother

Proband


Wild-type


Mutant

C

CDC45 Protein structure

N

DHH

NLS

CID

454

598aa

C

D

Wild-type

F96fs

E

HD1

HD2

HD3

CDC45

CDC45 65 kDa

Actin 42 kDa

Arbitrary Units

HD

CDC45
3.4 Validation of cellular damage from a *CDC45* variant.

Further characterization of the negative impact of this variant was required to better understand the mechanism leading to NK IEI. We first considered the cell cycle profile of BLCLs derived from the CDC45-deficient proband. Here, we found a decreased percentage of cells found in S phase compared to healthy donor, with a concurrent increase in the percentage of cells in G0/G1 phase (Fig 3.3A). Next, $\gamma$H2AX was assessed by both flow cytometry and imaging flow cytometry as a marker of DNA damage. These results were complementary in finding a significantly increased percent of cells positive for $\gamma$H2AX and an increased number of $\gamma$H2AX foci on a per cell basis (Fig 3.3B and C). CDC45 and RPA, including RPA70, RPA32, and RPA14, engage in a complex to facilitate binding onto single stranded DNA at replication forks, which supports our findings as CDC45-deficient cells would have a lowered capacity to bind with RPA (66). To this end, Western blotting with antibodies against polymerase-α, as a control, and RPA70 was conducted on chromatin-bound protein. Relative to healthy donor, the patient BLCLs displayed decreased RPA70 intensity while no changes were detected in pol-α (Fig 3.3D). Further, phospho-CHK1 was increased in proband BLCLs compared to healthy donor, indicating an increase in DNA damage signaling through ATR-CHK1 (Fig 3.3E). Lastly,
Nanostring gene expression data found an upregulation of pro-apoptotic genes in patient NK cells compared to three healthy donors (Fig 3.3F). Together, these data show a decrease in CDC45 functions and an increase in replication stress and DNA damage in cells that are CDC45-deficient, like resulting in apoptosis of patient cells as was indicated by Nanostring analysis.
Figure 3.3

A. DNA Content (7-AAD)

Cell incorporated BrdU

S phase 44.1%
S phase 21.8%

B. DAPI

γH2AX

HD
17.9%
31.0%

C. Brightfield DAPI γ-H2AX

HD

CDC45

D. Arbitrary Units

POLA 166 kDa
RPA70 70 kDa
H3 17 kDa

E. Arbitrary Units

pCHK1 56 kDa
Actin 42 kDa

F. Apoptosis Signaling

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<th>Fold Δ</th>
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<td>TRAF2</td>
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<tr>
<td>CHUK</td>
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</tr>
<tr>
<td>NFKB2</td>
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<td>NFKBIA</td>
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<td>NFKB1</td>
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</tr>
<tr>
<td>CASP8</td>
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</tr>
<tr>
<td>FAS</td>
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</table>
3.5 Activation of patient NK cells improves functionality

Cytokine-induced memory-like NK cells (CIML) have been increasingly used in the clinic to treat various cancers such as AML (179). This individual with a variant in CDC45 has consistently exhibited negatively-impacted NK cells with a lower percentage of mature CD56<sup>dim</sup> and adaptive NK cells in peripheral blood and decreased cytotoxic function. Here, we used various activation methods to stimulate patient NK cells to see if function improved. In a proliferation assay after 5 days of IL-15 (5 ng/mL) stimulation, we found that patient cells do indeed proliferate compared to no stim controls (Fig 3.4A). However, the proliferation profile appears smaller than that of the healthy donor, where only a single peak of late generational cells is visible, unlike the characteristic multiple peaks seen in the control. This may suggest cell death occurs in early generations of the proband’s NK cells. Next, CIML cytokines were utilized to further enhance NK cell functionality. Patient PBMCs given either low dose IL-2 or CIML cytokines maintained low viability compared to healthy donors (Fig 3.4B). The addition of low dose aphidicolin (0.5 µM) to induce replication stress decreased the percentage of viable cells from healthy donors, but not proband PBMCs, though this trend was not statistically significant.
Next, DNA damage was assessed by flow cytometry, where patient CD56\textsuperscript{bright} cells exhibited a higher percentage of positive $\gamma$H2AX cells (Fig 3.4C). Even when given low-dose IL-2 for survival, proband cells had more DNA damage compared to healthy donors. The $\gamma$H2AX level increased considerably when given CIML cytokines and further increased when given replication stress (Fig 3.4C). This trend was similar for CD56\textsuperscript{dim} cells given replication stress, however, there were no differences between healthy donor and CDC45 patient CD56\textsuperscript{dim} cells that were activated with CIML cytokines or only IL-2 (Supp Fig 3.1A). Finally, enriched patient NK cells were co-incubated with membrane bound IL-21 expressing 721.221 cells in media supplemented with IL-2 (200 U/mL) and IL-15 (5 ng/mL) to promote expansion and long-term survival in vitro (2). These expanded NK cells exhibited increased ssDNA as assessed by increased percentage of BrdU\textsuperscript{+} cells (Fig 3.4D), which would indicate increased replication stress. However, the functionality of these expanded NK cells was improved to be comparable to healthy donor. In a standard Cr\textsuperscript{51} release assay, there was no difference in percent specific lysis between CDC45-deficient expanded NK cells and healthy donor NK cells (Fig 3.4E). Additionally, phenotyping by flow cytometry confirmed increased granzyme B in patient expanded NK cells (Fig 3.4F), and found patient cells were comparable to healthy donor expanded NK cells for T-bet, EOMES, both transcription factors which aid in NK cell maturation and anti-tumor function (Fig 3.4G,H) (180). In addition, Ki-67, and pS6, indicators of proliferation and metabolism (181, 182), respectively, were found to be expressed similarly to healthy donor expanded cells (Fig 3.4I, J).

In this study, patient cells activated by various means, including IL-15 alone, CIML cytokines, and co-culture with membrane-bound IL-21 721.221 cells with soluble IL-2 and IL-15, can rescue NK cell function. Though viability remained low, replication stress and DNA damage remained elevated in activated patient cells. However, those cells that survive are able to
proliferate with rescued cytotoxicity, as well as exhibit improved metabolism through phospho-S6 and consistent developmental markers. Further research is needed to more thoroughly phenotype activated cells and test NK cell function and survival over time. However, the promising results of CDC45-deficient NK cells responding to activation displays clinical relevance and could indicate a potential pathway to treatment for NK IEI.
Figure 3.4

A. Normalized Proliferation (CFSE): HD IL-15 and CDC45 IL-15 compared to No Stim. HDIL-15 showed a higher proliferation rate of 93.8% vs. CDC45 IL-15 with 99.8% and No Stim with 6.44%.

B. % Viable Cells: HD IL-2, CDC45 IL-2, HD CIML, CDC45 CIML, HD CIML 0.5uM Aph, and CDC45 CIML 0.5uM Aph. HD IL-2 had the highest viability rate.

C. % H2AX+ Cells: HD NKG2A+CD57- and CDC45 NKG2A+CD57-. HD NKG2A+CD57- had an H2AX+ cell rate of 18.3%, while CDC45 NKG2A+CD57- showed 48.1%.

D. % BrdU+ Cells: HD and CDC45. HD showed an ns (not significant) value compared to CDC45.

E. % Specific Lysis: HD Expanded NK and CDC45 Expanded NK. HD Expanded NK showed higher specific lysis compared to CDC45 Expanded NK.

F. Normalized GzmB: HD Expanded NK and CDC45 Expanded NK. HD Expanded NK had a higher GzmB expression.

G. Normalized T-bet: HD and CDC45. CDC45 showed a higher T-bet expression.

H. Normalized EOMES: HD and CDC45. CDC45 showed a higher EOMES expression.

I. Normalized Ki-67: HD and CDC45. CDC45 showed a higher Ki-67 expression.

J. Normalized pS6: HD and CDC45. CDC45 showed a higher pS6 expression.
3.6 CDC45 deficiency modeling in an NK cell line.

Finally, modeling the CDC45-deficient patient using shRNA in an NK cell line was critical to supporting the mechanistic insights gained from studying this individual’s cells. Scrambled or CDC45-targeting shRNA containing GFP were transfected in YTS cells and selected using GFP sorting and puromycin. On western blot, 75% of CDC45 protein was knocked down (Fig 3.5A). We analyzed cell cycle with a particular focus on cells found in S phase. An increased percentage of CDC45-KD cells were found to be in S phase, with a concurrent increase in the percentage of cells in early S phase (Fig 3.5B). Interestingly, this phenomenon does not recapitulate the cell cycle profile of patient-derived BLCLs in Figure 3.3B, where there was a decreased percentage of CDC45-deficient cells in S phase. Next, we looked at the extracellular acidification rate (ECAR) measured by Seahorse assay to assess the glycolytic capacity of NK cells, an important indicator of enhanced proliferation and capacity for cytotoxicity when stimulated (183). Significant differences were found between scramble and CDC45-KD during the glycolysis challenge after the addition of glucose and again after the addition of oligomycin.
indicating increased glycolysis and glycolytic capacity in the CDC45-KD cells (Fig 3.5C). Next, we wanted to investigate the effect that knocking down CDC45 protein in YTS cells would have on DNA damage. Using confocal microscopy with staining against phospho-RPA and γH2AX, we found increased phospho-RPA foci and nuclear area in the CDC45-KD cells (Fig 3.5D and E). However, we found significantly decreased γH2AX foci in the knockdown cells, likely due to the role of CDC45 in DNA damage signaling (150) (Fig 3.5E). Finally, cytotoxic proteins were assessed by flow cytometry to indicate NK cell function in knockdown cells. Here, we found no changes in the percentage or fluorescence intensity of granzyme B+ (Fig 3.5F) or perforin+ (Fig 3.5G) cells compared to scramble controls. We also conducted bulk RNAseq on knockdown cells compared to scramble controls, and found very few differentially expressed genes (DEGs) (Supp Fig 3.2, Supp Table 3.1, Supp Table 3.2). Pathway analysis on DEGs did not highlight any areas of further interest. Modeling CDC45 deficiency in YTS cells supports the DNA damage and replication stress phenotype demonstrated by other helicase variants and the unique nature of
each helicase protein function, but leads to further questions as to the unique nature of CDC45 function.
Figure 3.5. CDC45 deficiency modeling in an NK cell line. (A) Validation of CDC45 knockdown was conducted by western blotting using anti-CDC45 and anti-actin antibodies. n=3 technical repeats. (B) Representative cell cycle plots with gating strategy based on scramble shRNA and quantification of the frequency of cells in S phase and early S phase. n=5 technical repeats. (C) Seahorse assay on 100,000 cells stimulated with IL-2 and IL-12 and plated with 2 µM oligomycin. ECAR was assessed at regular intervals over the course of 75 minutes. Student’s T test found statistical significance of p < 0.05 or p < 0.0005. (D) Representative images from confocal microscopy CDC45-KD or scramble shRNA YTS cells with DAPI staining and anti-phospho-RPA and anti-γH2AX staining. (E) Number of foci of phospho-RPA and γH2AX compared to scramble shRNA controls. Quantification of nuclear area in micrometers compared to scramble shRNA controls. (F) and (G) Intracellular flow cytometry for granzyme B and perforin. n=3 technical replicates. Representative histograms showing no differences in fluorescence intensity are shown with fluorescence minus one (FMO) controls as the dotted black lines. No statistical significance was found between CDC45-KD and Scramble control. Scramble, scramble shRNA YTS cells; CDC45, CDC45-KD YTS cells.
3.7 Discussion

Here, data was presented demonstrating the *CDC45* gene as a novel cause of NK IEI. The proband had a heterozygous, deleterious frameshift mutation likely resulting in nonsense mediated decay of the truncated mRNA. This individual displayed involvement of the NK cell compartment namely increased CD56<sup>bright</sup> cells in peripheral blood with decreased adaptive NK cells and decreased cytotoxic function, as well as other immune lineages such as CD20<sup>+</sup> B cells and CD4<sup>+</sup> T cells, contributing to IEI rather than the more narrowly-defined NKD. Deficiencies in the NK cell compartment was definitively determined by the recurrent severe viral infections, and it is interesting to note that the proband’s brother with the same variant in CDC45 was not susceptible to these infections. The proband’s brother also had NK cell numbers, including CD56<sup>bright</sup> and CD56<sup>dim</sup> populations, within reference ranges and cytotoxicity was intact. Speculation on this seemingly confounding finding is that environmental factors could influence the onset of NK cell deficiency or involvement in IEI. Thus, severe viral infections that the proband experienced in adolescence could have led to the manifestation of disease in the proband and not in other family members. Further, no other variants were identified that segregate to result in disease, although another variant not yet discovered cannot be ruled out.

In addition, CDC45 has distinct functions from the other helicase complex members, including DNA damage signaling through γH2AX, RAD53 and myc and recruitment of RPA to single-stranded DNA (66, 67, 150, 184). Our data suggests that there can be cell type specific requirements and functions for the CMG helicase complex, but that said, it is unknown why the B cells and CD4<sup>+</sup> T cells are affected but cytotoxic CD8<sup>+</sup> T cells are not. Modeling of CDC45 knockdown in YTS cells did not fully recapitulate the patient phenotype. The CDC45-KD cell line displayed replication stress and DNA damage signaling differences compared to controls,
namely through the increased RPA foci and nuclear area. We predict that DNA damage signaling is disrupted, since CDC45 signals to γH2AX during DNA damage (184) and our data found decreased γH2AX foci. However, knockdown of CDC45 in YTS cells contradicted the CDC45 proband-derived BLCL data and primary NK cell data. CDC45-KD cells had a different cell cycle profile, with an increased percentage of cells found in S phase whereas the proband’s BLCLs had half the percentage of cells in S phase compared to controls. We also found that perforin and granzyme B were unaffected and knockdown cells had increased glycolytic capacity, indicating increased proliferation and metabolism in general. Thus, the CDC45-KD cell line does not correlate with patient-derived BLCLs or NK cells on several points, and indeed bulk RNAseq data comparing the KD and scrambled cell lines found very few differences, with only 18 downregulated differentially expressed genes (Supp Fig 3.2, Supp Table 3.1, Supp Table 3.2). Immortalized cell lines may exhibit variable behavior compared to primary cells, utilizing compensatory mechanisms during protein knockdown. Primary cell studies using CRISPR-Cas9, shRNA, and siRNA repeated failed to generate stable clones with decreased CDC45 protein expression in our lab. However, work on induced pluripotent stem cells may be possible to give answers as to the developmental effects that occur with CDC45 deficiency.

Though this CDC45 variant is novel as a cause of NK IEI, this gene has been described to result in Meier-Gorlin Syndrome (MGS), also known as the “ear-patella-short-stature syndrome” (132, 185). Variants in CDC45 are biallelic and autosomal recessive in these cases and result in a distinctive phenotype with craniosynostosis and anorectal malformation, in addition to the typical triad of short stature, microtia, and no or under-developed patellae. No immune involvement has been reported to date in these patients. From study of the other CMG variants resulting in NKD such as GINS4 (26), we know that helicase variants themselves can account for
differing phenotypes. For example, the GINS4 probands with compound heterozygous variants had more severe susceptibility to viral infection than the parents with heterozygous variants, though it should be noted that only GINS2 and GINS3 have been described to result in MGS (186). More damaging variants can be expected to have a broader effect based on the ubiquitous function of the CMG complex. In the case of CDC45 resulting in NK IEI, the frame shift mutation is heterozygous, rather than biallelic, as is the case in individuals diagnosed with MGS. However, DiGeorge Syndrome, also known as 22q11.2 deletion syndrome, includes the deletion of a large suite of genes found on chromosome 22, often including CDC45 (187, 188). Immunodeficiencies and autoimmune diseases have been reported in these patients, although other genes found on chromosome 22 likely contribute to this effect (188, 189).

CDC45-deficiency has never been described to result in NK IEI to date. Here, we described a case study of a proband with severe recurrent viral infections, decreased adaptive cells, and increased CD56\textsuperscript{bright} cells in peripheral blood. Our understanding of NK cell biology and development grows with the study of new genetic determinants of human disease. Our findings of cytokine activation of the proband’s NK cells restoring cytotoxic functioning offers promising insights into potential treatments for NKD and NK IEI.
Discussion, Conclusions, and Future Directions

The principal activity of the CMG helicase is facilitating DNA replication while maintaining genomic integrity. Partial loss of function of helicase proteins, including MCM4, GINS1, MCM10 and GINS4, results in replication stress and DNA damage, ultimately leading to NKD. We have also described here a novel cause of NK IIEI due to loss of CDC45 protein, which also results in replication stress and DNA damage (Fig 3.3). In addition, this individual displayed cell cycle defects and increased phosphorylation of DNA damage repair protein, CHK1, in proband-derived BLCLs. CDC45 deficiency ultimately resulted in apoptosis, leading to a population-wise decrease in NK cells that led to this individual’s low cytotoxicity against target cells. Further, the development of adaptive NK cells was low to near absent in this individual, leaving her susceptible to recurrent viral infections (Fig 3.1). The study of adaptive cells in the context of helicase deficiency has never been done before, and indeed, is difficult to orchestrate in healthy donor models using chemical or genetic methods. It is compelling evidence that our CDC45-deficient individual lacks adaptive cells by flow cytometry, but further investigation into the requirement of helicase proteins in the development of adaptive cells would be highly illuminating. To this end, future work involving the use of HCMV sero-negative donors given replication stress and CMV peptide could provide information on the adaptive potential and survivability of NKG2C⁺ NK cells. CIML stimulation was selected for this study due to the ease of conducting the experiments using only cytokines, rather than stimulation involving co-culturing. Additionally, the recent increase in the use of CIML stimulation in the clinic and abundance of literature regarding the heightened effects on NK cells makes this approach relevant to human disease and reproducibility in the lab (39, 50, 176, 179). However, this system cannot be used indefinitely to culture primary NK cells in vitro and induces a lot of activation induced cell death.
(AICD). In our work, CIML cytokines served to activate primary NK cells into cell cycle and proliferation, as this is when the CMG complex would be most active. After confirming that CIML activation induced NK cells to cycle and proliferate (Fig 2.1B-C), we asked what happens when activated cells are given replication stress? Our results pointed to an apoptosis cascade when replication stress is present, lending important insight into sensitive NK cell biological responses when stress is encountered. Future work would include a caspase inhibitor to see if this natural apoptotic response to replication stress can be reduced. AICD could also play a role in NK cell responses and be compounded by the addition of replication stress, further resulting in cell death. Additional studies into the sensitivity of NK cells to AICD compared to other immune lineages would be of interest as AICD hints toward an NK cell sensitivity in the absence of replication stress. However, it is difficult to tease apart this result for a patient with a variant in a helicase protein, as these individuals would have replication stress from the start of NK cell generation rather than induction at a later point of maturity. Thus, our data shows that even if there is a developmental defect during helicase deficiency, some NK cells can mature and develop cytotoxicity, though some aspects of maturity may be impacted such as a generation of an adaptive pool.

For many primary immunodeficiencies, including SCID and NKD, an allogeneic hematopoietic stem cell transplant (HSCT), would be curative (190). However, HSCT is not a viable option for every case for a variety of reasons, including bone marrow conditioning complications and finding a matched donor (190). With regards to treatment of NKD and our work here, I propose that for individuals with some percentage of NK cells in peripheral blood, the use of CIML activation ex vivo could be therapeutically applied for preventative measures or in response to viral infection. This strategy is being tested currently to treat various blood cancers
However, in those individuals who lack NK cells in peripheral blood, a more permanent and direct option would target NK cell precursors or hematopoietic stem cells (HSCs), perhaps with the use of gene editing. In my hands, gene editing of helicase genes was unsuccessful in a number of experiments in both NK cell lines and primary NK cells isolated from peripheral blood of healthy donors. I attempted gene editing using electroporation to deliver lentivirus containing shRNA, siRNA or CRISPR-Cas9 against several helicase genes, including MCM10, CDC45, and GINS4. In the immortalized YTS cell line, protein knockdown was validated and data was generated to demonstrate the effects of helicase deficiency (Fig 2.6 and Fig 3.5). However, I found that prolonged time in culture, after about ~15 passages, cells began to compensate for helicase deficiency where doubling time increased and morphology became irregular. All data shown was generated during early passages. Another immortalized cell line, NK92, was more sensitive to helicase deficiency and would not recover after the electroporation. Primary NK cells from healthy donors proved to be incredibly sensitive to gene manipulation using these methods. After >15 attempts using various donors, genetic methods, and gene targets, I was unable to decrease helicase proteins due to cell death. Primary cells either could not recover from electroporation or they were very sensitive to helicase protein knockdown. Indeed, others have conducted genetic manipulation in primary NK cells with success (191, 192), so it is likely that helicase protein knockdown can be targeted using these experimental methods. However, gene editing targeting GINS4 was recently accomplished in iPSCs in my lab, which were then differentiated into NK cells (193). Genetic correction in earlier NK cell precursors or HSCs may ultimately prevent NKD or be used as a long term treatment plan for helicase-deficient individuals.

Using healthy donors to model the effects of replication stress provided useful insight into the mechanisms underlying NKD as a result of helicase deficiency. Initially, healthy donor PBMCs
must be prompted to proliferate through cytokine stimulation, using CIML cytokines. In vivo, a helicase-deficient individual would likely be exposed to various viral infections resulting in activation and proliferation of their cells. The increased proliferation of healthy donor PBMCs resulted in increased DNA damage, though not to the level of CDC45 proband NK cells (Fig 3.4C). The addition of replication stress in healthy donors using low dose aphidicolin then replicates the same endpoints found in helicase-deficient patients, namely apoptosis and decreased cytotoxic functionality. Importantly, this model allowed us to elucidate the mechanism of action resulting in these endpoints. Replication stress leads to increased p53 signaling and caspase activation in both NK cell subsets, as well as autophagy in CD56^{dim} cells. Those cells that survive replication stress have decreased cytotoxicity. These results parallel the phenotyping and damage assessment found in the CDC45 proband. However, when proband cells are stimulated using cytokines, we see a rescuing of the proliferation and cytotoxic potentials. An analogous improvement would be difficult to generate in our healthy donor model given that they are already stimulated and experiencing AICD. Further work could re-stimulate the surviving CIML healthy donor primary cells, though the time in culture may preclude long-term results as AICD and exhaustion may occur. Likewise, it is unknown if CIML or other activation methods could be a potential therapeutic option in helicase-deficient individuals until further investigation considers AICD or exhaustion in the long term. Helicase proteins clearly play a critical role in human natural killer cells, thus understanding the role of the CMG helicase through the lens of NKD contributes to our knowledge of NK cell development and function through the sensitive threshold required of NK cells compared to T cells.

Primary cell models are critical to gaining a stronger understanding of not only NKD, but NK cell biology in general. This dissertation features the use of shRNA to knockdown helicase
protein expression in a YTS cell line compared to a scramble shRNA control. YTS cells are derived from pericardial effusion of an NK cell lymphoblastic leukemia/lymphoma and were transformed using Epstein-Barr virus (170). Thus, relying on these transformed models do not fully reflect the consequences of helicase deficiency. For example, we cannot assess adaptive cells using a cell line, and in the case of the CDC45-KD cells, the cell cycle profile was not reflective of the CDC45 proband-derived BLCLs. The CDC45-KD cells also displayed decreased γH2AX foci, while proband-derived BLCLs had more γH2AX foci compared to a healthy donor. However, important supportive data was gained from knocking down MCM10 and CDC45 in this cell line, including the reproducibility of DNA damage and replication stress and poor survival. It is important to note that these proteins resulted in differing phenotypes, particularly in the assessment of γH2AX foci, which may point to the different roles that these proteins play individually or may be a feature of transformed cells that undergo transfection and a further antibiotic selection process. All this being said, the use of primary cells is important to gaining a stronger understanding of the immune system and NK cell development. Using primary PBMCs can help to account for biological heterogeneity and future work would include testing additional donors. Primary cells from peripheral blood have also allowed us to investigate the more mature subsets of NK cells and the subset-specific consequences of helicase deficiency. The Mace lab investigated immature subsets during helicase deficiency in the context of GINS4 deficiency in iPSCs (193). Additional work should seek to understand larger commonalities between other helicase proteins and the CMG complex as a whole during development of more immature NK cell subsets.

Why NK cells are particularly affected by helicase deficiencies

It is still not fully understood why natural killer cells are the primary immune cell subset that
is affected by helicase variants in IEI. Proposed mechanisms for this unusual susceptibility include a particular requirement for proliferation in NK cell terminal maturation from the CD56\textsuperscript{bright} to CD56\textsuperscript{dim} stage (26-30). However, this has not been formally demonstrated, and the limited availability of material from rare individuals has made this hard to test. While N KD is a common feature of individuals with helicase deficiencies, there are cell type-specific effects manifested in the various clinical and cellular phenotypes of the disease. All variants reported to date are partial loss-of-function due to heterozygosity, and in GINS1 deficiency, the severity of damage by different variants correlated with the clinical severity of the disease, suggesting a dose-dependent effect of the availability of GINS1 protein (29). This, combined with the tight regulation of CMG protein expression, suggests that there may be a dose-dependent window in which NK cells are affected while sparing other immune lineages. Perhaps more severe variants would affect other lymphoid cell subsets, a hypothesis that is supported by the T cell lymphopenia reported in some patients (85, 86). While still speculation at this point, here we summarize this and other potential models for the effect of limited helicase function on NK cell development or homeostasis. We propose that CMG variants could impact NK cells in three potentially overlapping mechanisms; namely, by dysregulating mature NK cell subset homeostasis, by impacting NK cell terminal maturation, or by introducing lineage bias at earlier stages of hematopoiesis (Fig 1.3). Alternatively, mechanisms of DNA fitness related to induction of DNA damage pathways, in the context of hypomorphic RAG phenotypes, may contribute to NK cell specific effects.
As the underlying mechanism of NK cell dysfunction in helicase deficiencies is still unclear, it is not known what underlies the viral susceptibility identified in these individuals. However, given that deletion of MCM10 in an NK cell line had no direct effect on NK cell cytotoxic function (27), it is most likely that impaired NK cell development results in both fewer numbers of effective NK cells and intrinsically impaired NK cell function on a single cell basis. While this mechanism is likely not specific to helicase deficiencies and instead applies whenever NK cell function or numbers are compromised, a greater understanding of the true nature of impaired NK cell development in the context of helicase deficiency will help better connect the NK cell phenotype with clinical phenotypes.

In addition to increased susceptibility to viral infections, NKD confers an increased risk of cancer associated with viruses such as EBV, though the direct impact of NKD on cancer incidence...
is largely unstudied (194). While no individuals with NKD resulting from helicase mutations have described malignancies, this may be due to the small cohort of patients described to date. Several helicase protein deficiencies have been associated with genomic instability, a hallmark of cancer, in patient cells and through experimental modeling. Namely, MCM10 deficiency resulted in increased replication stress which may contribute to genome instability and telomere erosion, and GINS1 deficiency leads to abnormal nuclear structures and investigation of MCM4 deficiency found chromosome breaks (27, 29-31). This is bolstered by in vivo data from the mouse model Mcm4<sup>Chao3</sup>, which displayed high levels of chromosomal instability with increased micronuclei and the development of mammary adenocarcinomas (89). Similarly, deficiency of MCM2 in a mouse model displays the development of lymphomas and shortened lifespans (195). Conversely, the overexpression of virtually all helicase protein components have also been found in cancer, details of which are outside the scope of this review (51). The small cohort of patients with helicase deficiency makes it difficult to understand if there is increased susceptibility to cancer and the mechanisms underlying this phenomenon.

The most intuitive mechanism to explain an NK cell specific effect of these variants is that committed NK cell progenitors rely more acutely on DNA replication machinery than other cell types. Alternatively, it is possible that NK cells are more poised to undergo apoptosis in response to stress, where as other cell types have more resiliency and undergo repair mechanisms instead. However, as mentioned before, NK cells proliferate more rapidly than T cells in the absence of infection (111). Patients with PNH with somatic PIGA mutations clearly demonstrate that CD56<sup>bright</sup> NK cells and neutrophils are the two most proliferative hematopoietic populations (120). Congruently, a frequent immune phenotype that occurs along with NKD is neutropenia (Table 1.1). These data suggest that the variants are dysregulating homeostasis of the most
proliferative cell types. Further supporting this idea are variants found in DNA synthesis related genes outside of the CMG helicase, \textit{POLA1} and \textit{POLE2}, which also manifest in NKD and other common CMG variant phenotypes (87, 88, 127). In a parallel example, a variant in \textit{RTEL1}, a gene involved in helicase activity during telomere elongation, can also result in decreased NK cells in PBMC (139). The converging phenotype of NKD in variants of S phase related genes demonstrate that cell cycle dysregulation is an important mechanism. This effect could be due to differential proliferative kinetics or differential regulation of replisome protein expression. Further studies, including epigenetic analysis and the use of FUCCI cell cycle labeled cells, must be carried out to understand what makes NK cell proliferation more vulnerable to these changes.

The PNH example with somatic mutation highlights that in NK cells from adult peripheral blood, CD56\textsuperscript{bright} cells, but not the CD56\textsuperscript{dim} cells, are actively proliferating. In many of the NKD patients, whose variants are germline mutations, both CD56\textsuperscript{bright} and CD56\textsuperscript{dim} populations are affected, suggesting additional mechanisms for subtype specific cellular fitness that may arise throughout development. The decrease of CD56\textsuperscript{dim} NK cells in peripheral blood suggests that CD56\textsuperscript{dim} cells are not being produced as effectively in patients with helicase deficiencies, and in vitro and humanized mouse models of patient cells suggest that NK cell terminal maturation is affected (26, 27). In this model, helicase defects would manifest most prominently during the final steps of NK cell maturation. For example, MCM4 is more highly expressed in CD56\textsuperscript{bright} NK cells, so its insufficiency results in DNA damage and may ultimately reduce, but not completely block, maturation into the CD56\textsuperscript{dim} subset (30). Alternatively, diminishment of mature NK cells may reflect the normal generation but impaired survival of a specific CD56\textsuperscript{dim} subset. The previously described differential survival and turnover of adaptive NK cells could support a model in which adaptive NK cells, or some other subset, were either selectively retained or selectively impaired.
The idea that NK cell differentiation is unaffected up until terminal maturation is challenged by the decrease in absolute number of circulating NK cells in addition to the change in CD56$^{\text{dim}}$ to CD56$^{\text{bright}}$ ratio found in most affected individuals and the reduced frequency of ILC precursors in GINS1 patients (29). Current evidence on adjacent lineages which can shed light on more developmental phenotype of NKD, such as ILC and unconventional T cells, is limited to only a few patients. Study of the GINS1 variants with decreased ILC and GINS4 variants with unaffected T cells, including γδ T and MAIT cells, suggest that the effects are more specific to NK/ILC common precursors. Further investigation on how other helicase variants affect ILCs and all T cell lineages would be important to understand the lineage bias more comprehensively. However, these cell types are fundamentally tissue-resident, making the accessibility a major challenge. An in vitro or animal model system may be needed to understand these lineage biases with sufficient resolution.

While it is more difficult to analyze the requirements for early human NK cell differentiation due to its localization in tissue, we can consider the effects of helicase deficiency on hematopoiesis and early innate lymphoid cell differentiation. One potential mechanism by which helicase variants could affect NK cells during hematopoiesis would be skewing of lineage biases in hematopoietic precursors or hematopoietic stem cells (HSCs). In mice, delayed cell cycle progression has been observed in HSCs from aged mice, accompanied by lower MCM4 and MCM6 expression and impaired HSC functionality (196). Along with decreased cell cycle, many groups have reported that aged HSCs have a decreased potential for lymphoid lineages while myeloid potential is intact (197-200). This suggests that decreased CMG expression, including in aging, may position HSCs to be biased against NK cells and other innate lymphoid cells that require continuous regeneration. That said, aging is a complex process that manifests in additional
aspects of HSC lineage output, including DNA damage, clonal selection, epigenetic drift, and polarity shift (201). Therefore, further studies must be done to understand how the phenotypes and mechanisms of aged HSCs compare to those of HSCs with helicase variants.

One could speculate that a relatively mild lineage bias in HSCs could be combined with additional defects in proliferation or cell cycle regulation that may be a feature of later stages of NK cell development, leading to the dysregulated NK cell subsets found in the peripheral blood of patients. Further, such a model could also account for what seem to be environmental factors that can contribute to clinical severity, most notably in the GINS4 cohort where the most clinically affected sibling had a significant CMV infection early in life (26). Disruption of stem cell or precursor homeostasis due to severe infection is reminiscent of the effect of recurrent inflammation on HSC function and could link triggering viral events with subsequently limited NK or innate lymphoid cell lineage potential (202). However, the question remains of why NK cells are most affected and why patients don’t exhibit signs of other HSC dysregulation, such as bone marrow failure. Careful manipulation of helicase protein function, combined with molecular studies that link HSC function to downstream innate lymphoid lineage potential, will be necessary to answer these questions.

Given the decreased numbers of NK cells in circulation and the rarity of affected individuals, many studies described above have used patient or control fibroblasts, CD34+ cells, NK cell lines, or B cell lines to understand the mechanisms of helicase variants in cell cycle and DNA damage. However, as shown by disease phenotypes, the effects of the helicase variant appear to be cell type specific and potentially affecting multiple steps throughout hematopoiesis. Human induced pluripotent stem cells (iPSCs) provide a unique opportunity to model the pathogenesis of such diseases in an NK cell specific way. Several studies provide methods for NK cell generation
by modeling embryonic or postnatal NK cell development (203-205). Utilizing patient-derived iPSCs is one tractable model to circumvent technical hurdles associated with the study of specifically human immunity and address questions of lineage specification, albeit one that makes it difficult to study mature NK cell homeostasis and generation of adaptive NK cells (193).

Finally, we can consider the non-canonical roles of helicase proteins. Multiple helicase components are involved in different aspects of regulating replication, including genomic stability, DNA damage signaling, and replication fork stalling. Additionally, most helicase proteins have alternative binding partners outside of the CMG helicase, which could account for the variability between helicase variants seen in these NKD patients and in vitro studies. While similarities between patient and cellular phenotypes between multiple CMG protein deficiencies suggests that replisome dysfunction is at the heart of NK cell deficiency, better modeling of different aspects of canonical and non-canonical CMG protein functions can be informative of both NK cell biology and the role of replisome function in immune cell generation, homeostasis, and disease.

**Summary Figure.** Activation of NK cells by cytokine stimulation or viral infection results in DNA damage and replication stress. From here, cells can undergo apoptosis through increased p53 signaling and caspase activation. Those cells that survive exhibit decreased cytotoxicity against target cells and both of these pathways downstream of replication stress contribute to natural killer cell deficiency.
Methods

Cell isolation and cell lines

Blood from healthy donors was collected under the guidance of the Institutional Review Board at Columbia University and in accordance with Declaration of Helsinki guidelines. PBMCs were isolated by density gradient centrifugation from the buffy coat of healthy donors from the New York Blood Bank using Ficoll-Paque (Fisher Sci Cat# 45-001-750). PBMCs were stored frozen in 90% FBS 10% DMSO and thawed for each experiment. Once thawed, PBMCs were resuspended and maintained in RPMI 1640 medium (Thermo Cat# 11875135) supplemented with 10% heat-inactivated human AB serum (GeminiBio Cat# 100-512), 1% penicillin-streptomycin (PenStrep; Thermo Cat# 15140163), 2mM Glutamax (Thermo Cat# 35050079), 1X non-essential amino acids solution (NEAA) (Thermo Cat# 11140076), 1mM sodium pyruvate (Thermo Cat# 11360070), and 1M HEPES (Thermo Cat# 15630130). YTS cell lines, which are derived from malignant NK cell leukemia/lymphoma (170), were maintained with the same media as PBMCs, except with 10% FBS (Atlanta Biologicals Cat# S11150) instead of human serum. K562 cells were maintained in RPMI medium supplemented with 10% FBS, 1% PenStrep, and 2mM GlutaMAX at 37°C and 5% CO₂. All cell lines were confirmed negative for mycoplasma.

NK and T cells Stimulation
Frozen PBMCs were thawed and briefly rested in human serum-supplemented RPMI medium. To generate activated NK cells, cytokines were added to the media (IL-15 100 ng/mL, IL-18 50 ng/mL, IL-12 10 ng/mL) for overnight stimulation. PBMCs were then washed three times and resuspended with fresh media and low dose IL-2 (20 U/mL) for expansion phase in a round-bottom 96-well or 24-well plate. Aphidicolin (0.25 µM, 0.5 µM, 1 µM, or 5 µM) (Sigma Cat# A4487) was also added at this time. To generate activated T cells from PBMCs, fresh media was supplemented with 30 U/mL of IL-2 and a 1:1 ratio of anti-CD3 and -CD28 Dynabeads (Thermo Fisher Cat# 11131D) and similarly treated with aphidicolin. Cells were harvested one or three days after aphidicolin and used for flow cytometry with surface markers to identify NK and T subsets or enriched using StemCell Negative Isolation kits (EasySep Human NK Cell Isolation Kit StemCell Cat# 17955 and EasySep Human T Cell Isolation Kit StemCell Cat# 17951).

**Flow cytometry**

Phenotypic flow analyses were conducted on both PBMCs and cell lines using multicolor flow cytometry. Cells were washed in PBS then surface stained with fluorochrome-conjugated antibodies and viability dye at 4°C for 20 minutes while protected from light. Cells were washed again then fixed and permeabilized for intracellular staining using the FoxP3 nuclear staining kit (Fisher Scientific Cat# 00-5523-00) according to manufacturer’s instructions. Fluorescence minus one and unstained controls were included for all experiments. To measure IFN-γ, 10 µg/mL Brefeldin A (Biolegend Cat# 420601) was added during stimulation to inhibit protein transport. Data were acquired on a BD Fortessa and analyzed with FlowJo v.10.9.0 (BD Biosciences).
Cell cycle analysis

Cell cycle analyses were conducted with the BrdU assay kit from BD Biosciences according to manufacturer’s instructions. Cells were incubated with 10 µM BrdU for two hours followed by extracellular marker staining, fixation, denatured with DNase, and incubation with anti-BrdU (BD Biosciences Cat# 559619) and any other intracellular markers. Finally, cells were stained with 7-AAD for five minutes, then analyzed on a BD Fortessa cytometer. Results were analyzed with Flow Jo and the frequency of cells found in cell cycle phases (G1/G0, S, G2/M) was determined by gating BrdU-7-AAD intensity.

Confocal microscopy

Cells were briefly adhered to #1.5 imaging chambers followed by fixation and permeabilization using Cytofix/Cytoperm (BD Biosciences Cat# 554714). Immunostaining was performed with phospho-RPA (Thermo Cat# PA5-104809) followed by goat anti-rabbit Alexa Fluor 488 (Thermo Cat# A32731), then γH2AX Alexa Fluor 647 (BD Biosciences Cat# 560447). DAPI staining was performed for 10 minutes prior to imaging. Images were acquired with a 100X 1.46 NA objective on a Zeiss AxioObserver Z1 microscope stand equipped with a Yokogawa W1 spinning disk by imaging cell volumes with a 0.17 um Z step size. Illumination was by 405 nm, 488 nm, and 647 nm solid state lasers and detection was by a Prime 95B sCMOS camera. Data were acquired in SlideBook software (Version 6, Intelligent Imaging Innovations) and exported as OME-TIFF files for further analysis. Images were analyzed as Z projections in Fiji (206) using the “Analyze Particles” plug-in with a minimum size of 0.05 um2. Data were exported to Prism 9 (GraphPad Software) for graphing.
**NK: Target Conjugate Imaging**

NK cells were briefly co-cultured with K562 target cells for 15 to 45 minutes in an eppendorf tube, then gently transferred to a PLL-coated microscope slide. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences Cat# 554714) and then immunostained for anti-CD56, phalloidin, anti-Perforin, and anti-alpha tubulin in the microtubule organizing center (MTOC). Using mounting medium without DAPI, coverslips were place and allowed to cure overnight. Images were acquired as described above and analyzed using Fiji.

**shRNA Lentiviral Transduction**

YTS cells were maintained in culture medium in growth phase and $10^6$ cells were plated in 6-well plate 24 hours prior to shRNA viral transduction. Cells were washed with sterile PBS and resuspended in 400 µL fresh complete media with 100 µL TransDux MAX Lentivirus Transduction Reagent (SystemBio Cat# LV860A-1). Viral supernatant for non-targeting (SMARTvector Lentivirus shRNA hEF1a TurboGFP, Horizon Discovery Cat# S02-005000-01), MCM10 (SMARTvector Lentivirus shRNA MCM10 TurboGFP, Horizon Discovery Cat# V3SH7669) or CDC45 (SMARTvector Lentivirus shRNA CDC45 TurboGFP, Horizon Discovery Cat# V3SH7590) were added to respective wells and the plate was spun at 1500g for 90 minutes at 35°C. Cells were then incubated for 48 hours at 37°C. Antibiotic resistance selection with puromycin began 72 hours after transduction starting at 0.5 µg/mL and gradually increasing to 2 µg/mL over the course of two weeks. Cells were GFP+ sorted after 2 µg/mL puromycin selection. MCM10 and CDC45 protein knockdown was validated by western blot.

**DNA fiber analysis**
DNA fiber analysis was carried out as previously described (207). Briefly, cells were incubated with 25 μM IdU for 30min, washed 3 times with warm PBS and incubated with 25 μM CldU for another 30min. Fibers were stretched on slides and stained with CldU (Biorad Cat# OBT0030), and IdU (BD Cat# 347580) antibodies. Slides were mounted with ProLong Gold Antifade mounting medium and dried overnight. The fiber tracks were imaged on a Nikon Eclipse 90i microscope fitted with a PL Apo 40X/0.95 NA objective and measured using ImageJ software.

**Proteomics analysis**

NK cells and T cells were stimulated using IL-15, IL-12, and IL-18 or anti-CD3/CD28 beads with IL-2 respectively and harvested three days after stimulation. Magnetic Dynabeads were removed from T cell samples using the DynaMag-2 Magnet (Invitrogen Cat# 12321D) and then NK and T populations were enriched using StemCell Negative Isolation kits. Cells were briefly washed with cold PBS twice and transferred to 1.5mL eppendorf tubes. Samples were flash frozen using liquid nitrogen and delivered to the Proteomics and Macromolecular Crystallography core at the Columbia University Herbert Irving Comprehensive Cancer Center (HICCC). Label-free protein quantification using LC-MS was conducted on the samples. Data was analyzed using Microsoft Excel and graphs were made with Prism 10.0.0 (GraphPad).

**NK cell cytotoxicity assays**

Cytotoxicity of enriched activated NK cells from PBMCs from five healthy donors against K562 erythroleukemia target cell line was performed by four-hour Cr$^{51}$ release assay. Briefly target cells were incubated with Cr$^{51}$ radionucleotide (Perkin Elmer, NEZ030S001MC) for one hour, washed and incubated with NK cells at increasing effector to target ratios, or in the absence of
effectors for spontaneous release controls, in triplicate at 37C for 4 hours. After 4 hours, total experimental release controls were lysed with 1% octylphenoxypolyethoxyethanol. Plates were spun and the supernatant was transferred to a LumaPlate (PerkinElmer), dried overnight, and read in a gamma scintillation counter. Total lysis and spontaneous lysis controls were included. Percent specific lysis was calculated using the following formula: 

\[
\frac{(\text{experimental release}-\text{spontaneous release})}{(\text{total release}-\text{spontaneous release})} \times 100.
\]

**Western blotting**

Cells were lysed in RIPA buffer (Thermo Cat# 89900) with 1X Halt Protease and Phosphatase Inhibitor cocktail (Thermo Cat# 78443). Cell lysates were separated by gel electrophoresis using a 4%-12% gradient cell (Thermo Cat# NW04120BOX) or 12% gel (Thermo Cat# NW00127BOX) and then transferred to a 0.2 µm pore-size nitrocellulose membrane (Thermo Fisher Cat# LC2000). The membranes were blocked using 10% nonfat dry milk solution (Fisher Scientific Cat# 50-488-786) in PBS-tween 0.1%, then incubated with primary antibodies in a 5% BSA solution. Membranes were imaged on the LiCOR CxL and analyzed using the ImageStudioLite software (version 5.2.5).

**Nanostring RNA**

Patient and three healthy donor NK cells, isolated from whole blood, were submitted for multiplex gene expression analysis of about 500 human immune-related genes to Nanostring. Total RNA was isolated using manufacturer’s instructions (Macherey-Nagel Nucleospin RNA XS Cat# NC0389511) and flash frozen with liquid nitrogen. Data were analyzed with Loupe Browser 5.0 and pathway analysis conducted using PANTHER.
RNA sequencing

Bulk RNA sequencing of cell lines was conducted by the Columbia University Genome Center. Total RNA was submitted, prepared using the Macherey-Nagel kit, and run on the Illumina NovaSeq 6000 instrument. A STRPOLYA library was created using poly-A pulldown at a depth of 40 million reads. Data was analyzed using iDep 93 and pathway analysis conducting using PANTHER.

Seahorse assay

YTS cell lines, scramble and CDC45-KD, were maintained as described above and frozen in 90% FBS 10% DMSO. Cells were shipped on dry ice to collaborators at University of Chicago, where they were thawed and stimulated with 20 ng/mL IL-2 and 10 ng/mL IL-12 for 18 hours prior to analysis. 100,000 cells were plated with 2 μM oligomycin, an inhibitor of mitochondrial ATP synthase to promote glycolysis. The Agilent Seahorse XF Glycolysis Stress Test Kit (Agilent Cat# 103020-100) was used to test the extracellular acidification rate (ECAR). Figures were made using Graphpad Prism.

Imaging flow cytometry

The Amnis ImageStreamx MkII, which combines the throughput and single cell resolution of flow cytometry with the detailed information generated by microscopy, was used for γH2AX spot counting. The ImageStream is equipped with four excitation lasers for fluorescence detection (405 nm, 488 nm, 561 nm, and 642 nm) with adjustable powers and a fifth laser in the infrared portion of the spectrum for side scatter measurements. Images of cells
in flow are captured in 12 twelve channels spread equally across two cameras, each equipped with a dedicated brightfield channel synchronization between the two cameras. A total of 10 fluorescence signals can be measured in a single sample along with brightfield images. The instrument is equipped with three objectives – 20x, 40x, and 60x – to accommodate cells of various sizes and a variety of different applications. Data was acquired through INSPIRE software and analysis was performed using INSPIRE.

Statistics

Statistical analyses were conducted using Prism 10.0.0 (GraphPad). All data show mean ± SEM. P value ≤ 0.05 was considered significant. One-sample 2-tailed Student’s t test was used to compare the mean of an experimental condition to the control, normalized to one. One-way ANOVA was used to compare multiple conditions. Values were statistically non-significant when P>0.05. P values on graphs are represented as: * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.
Appendix A
Supplemental Figure 2.1: Enrichment of NK cells by CIML activation and validation of T cell activation. (A) Percentage of CD56⁺CD3⁻ NK cells enriched by CIML activation compared to no prime controls. (B) Percentage of activated CD25⁺CD69⁺ T cells. Gating is based on no stimulation controls for CD4⁺ or CD8⁺ cells, respectively.
Supplemental Figure 2.2: Etoposide impacts NK cells similarly to aphidicolin. (A) Percentage of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} cells after CIML stimulation with addition of etoposide. Ratio of bright to dim cells was calculated. (B) Percentage of γH2AX cells in CD56\textsuperscript{bright} and CD56\textsuperscript{dim} cells. Representative flow dot plots shown for the lowest concentration of ET. (C) Percentage of phospho-p53 Serine 15 positive cells in CD56\textsuperscript{bright} and CD56\textsuperscript{dim} cells with representative flow plots for the lowest concentration of ET. n=4 healthy donors and One-way ANOVA used to calculate statistical significance on all graphs.
Supplemental Figure 2.3: p21 analysis of activated NK vs T cells with and without replication stress. (A) Fluorescence intensity of p21 was collected for CD56$^{\text{bright}}$ and CD56$^{\text{dim}}$ NK cells and CD3$^+$ T cells. n=4 biological replicates.
Supplemental Figure 2.4: Cell cycle analysis during NK cell activation with replication stress.

(A) Representative flow plots of cell cycle gating for C1ML stimulation with and without low dose aphidicolin. Quantitative analysis of percentage of cells found in S phase. n=4 healthy donors One-way ANOVA calculated statistical significance.
**Supplemental Figure 2.5: Gene expression of helicase and replication-related genes. (A – F)**

Relative expression in arbitrary units for RNA abundance of given helicase proteins and replication genes was collected and analyzed from BioGPS. Significance was determined using a One-way ANOVA.
Supplemental Figure 3.1: DNA damage in stimulated CDC45-deficient CD56\textsuperscript{dim} cells in response to replication stress. CD56\textsuperscript{dim} NK cells do not have increased DNA damage when activated by CIML cytokines like the CD56\textsuperscript{bright} subset. γH2AX only increased with low-dose aphidicolin in the CDC45-deficient patient.
Supplemental Figure 3.2: Bulk RNAseq on CDC45-KD and Scramble YTS Cell line.
(A) PCA plot identified 42% of variance between CDC45-KD and Scramble cell lines. 
(B) Heatmap visualizes the top up- and down-regulated differentially expressed genes between the two cell lines. 
(C) Volcano plot of the differentially expressed genes between Scramble and CDC45-KD cell lines.
Supplemental Table 3.1: CDC45-KD vs Scramble YTS Bulk RNAseq
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Supplemental Table 3.2: CDC45-KD vs Scramble YTS Bulk RNAseq upregulated differentially expressed genes.

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