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INVITED REVIEW

ITAM-based receptors in natural killer cells

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Summary

The ability of cells of the immune system to acquire features such as increased longevity and enhanced secondary responses was long thought to be restricted to cells of the adaptive immune system. Natural killer (NK) cells have challenged this notion by demonstrating that they can also gain adaptive features. This has been observed in both humans and mice during infection with cytomegalovirus (CMV). The generation of adaptive NK cells requires antigen-specific recognition of virally infected cells through stimulatory NK receptors. These receptors lack the ability to signal on their own and rather rely on adaptor molecules that contain ITAMs for driving signals. Here, we highlight our understanding of how these receptors influence the production of adaptive NK cells and propose areas in the field that merit further investigation.

KEYWORDS
adaptive NK cells, adaptor molecules, ITAM, memory NK cells, natural killer cells, NK receptors

1 | INTRODUCTION

Organisms are under constant pressure to evolve mechanisms to protect themselves from pathogens in their environment. This is evident from the vast arrays of receptors and specialized cells that the immune system employs to detect and neutralize threats. The innate immune system serves as the first line of defense against threats from pathogenic cells.

Natural killer (NK) cells are group 1 innate lymphoid cells (ILC) employed by the immune system that are critical for recognizing pathogenic cells. These include transformed cells, virally infected cells, or antibody-coated cells.[1,2] NK cells express a myriad of cell surface receptors that can be categorized as being either stimulatory (activating) or inhibitory. The stimulatory receptors come from families of receptors that have been under extreme pressure to adapt to rapidly evolving ligands. This pressure has resulted in the separation of the signaling chains (adaptors) from the ligand recognition chains (receptors), thereby accommodating the rapid diversification of extracellular domains to adapt and recognize stress-induced ligands while maintaining conserved signaling domains. Both adaptive and innate receptors exhibit this multicomponent feature. This ability to facilitate the diversification of extracellular chains while retaining the ability to associate with signaling adaptor chains is a result of tightly conserved transmembrane (TM) domains on both receptors and adaptors.[3,4] Generally, these adaptor molecules contain an acidic residue in the TM domain that, in the plasma membrane, attracts a basic residue in the TM on the receptor. In addition to the TM domain, most intracellular domains of adaptors contain the immunoreceptor tyrosine-based activation motif (ITAM), which is identified as (D or E)xx**Y**xx(L or I)$_{X6\text{-}8}$**Y**xx(L or I) where X designates any amino acid and X6-8 denotes six to eight amino acids between the **Y**xx(L or I) residues.[3] Activation through the receptors results in phosphorylation of the tyrosine residues in the ITAMs by Src family kinases, resulting in the recruitment of the ζ-associated 70 kDa

(ZAP70) or Syk kinases that drive downstream signal transductions. Upon activation, NK cells launch cytotoxic granules containing perforin and granzymes towards a target cell, resulting in loss of cellular integrity and apoptosis of the pathogenic cell.

1.1 | Outstanding questions in the field

Despite our advances in understanding NK cells, we still do not fully understand many aspects of how NK cells develop memory and what role ITAM-based receptors play in this process. For example, does affinity of receptor-ligand interaction influence generation of memory? How important are the ITAM adaptors in generating memory? Does increasing the number of ITAMs associated with a receptor influence the generation of memory? To what extent do elements in the TM domain of adaptors and receptors influence activation? Do elements in the cytoplasmic domain of receptors and adaptors influence the type of signal transmitted upon activation? Is ITAM signaling important for maintaining memory NK cells? What are the key signaling events that lead to the formation of memory in NK cells? Is there synergy with other signals, such as adhesion molecules, co-stimulatory, or inhibitory signals? What generates longevity in NK cells? How does cytokine activation synergize with or influence ITAM-mediated signals? Do memory NK cells behave differently in the tissues than they do in circulation? What are the specific stimuli that allow the formation of memory NK cells? How do different kinases mediate differences in signal cascades? Are there specific receptors that can drive the formation of memory in NK cells? Are there specific adaptors that contribute to the generation of adaptive NK cells, or is this a feature of all ITAM-based adaptors? Can these adaptors drive these mechanisms in other cell types? Ultimately, these questions will lead us to the critical question: Can we engineer adaptive NK cells? If so, can we design them for enhanced effectiveness against specific targets?

In this review, we will provide an overview of the ITAM-associated NK receptors in NK cells, discuss recent mechanisms of their activation, and highlight their contributions to generating memory while underscoring gaps in knowledge. The goal is to provide an overview of how much the field has advanced, enabling us to pose the many questions about the role of ITAM-associated receptors in the generation of memory. In this article, we will refer to memory or adaptive NK cells interchangeably.

2 | ITAM-CONTAINING MULTICOMPONENT RECEPTOR ASSEMBLY AND SIGNALING

Multicomponent receptors are composed of several single-pass transmembrane proteins, a ligand-binding subunit, and a dimeric signaling subunit that must assemble for proper receptor function. This assembly is confined to the membrane since the ligand-binding subunit exists primarily in the extracellular space with a minimal intracellular region, while the signaling subunit has a minimal extracellular portion and robust intracellular ITAM region. For most multicomponent receptors, assembly is mediated by an amino acid motif composed of two acidic residues (aspartic acid) on the dimeric signaling subunit and one basic residue (lysine or arginine) on the ligand-binding subunit. This 2-to-1 stoichiometry of subunits ensures that for every ligand-binding subunit, there are multiple ITAMs available for signal propagation. Additionally, this 2-to-1 binding motif is notable because three polar and ionizable residues are confined to assemble in the nonpolar membrane and employ a complex network of hydrogen bonds, van de Waals interactions and electrostatic interactions that ensures stable assembly. Assembly is localized to the 2-to-1 motif, which retains a locally stable conformation.[5] In the absence of one of the three polar residues through mutation, the local conformation is disrupted, and assembly and therefore signaling are abrogated.[6,7] Despite the conformational stability of the 2-to-1 polar residue motif, a globally dynamic arrangement of the protein helices has been observed in the NKG2C-DAP12 receptor complex.[5] This globally dynamic arrangement provides insight into how DAP12, a highly promiscuous signaling subunit, is able to associate with so many ligand-binding subunits so long as they contain a basic residue in their transmembrane region. This finding also provides insight into how other adaptor molecules may accommodate multiple receptors with conserved, yet unique TM alpha helices.[3,5] However, some multicomponent receptors do not rely on a 2-to-1 binding motif for assembly, as is the case for CD16. Instead, assembly in this receptor relies on a complex network of polar and nonpolar residues[8] that is highly sensitive to even very conservative mutations (discussed below). Nonetheless, one commonality among all multicomponent receptors is that, in the absence of receptor assembly, extracellular signal does not result in ITAM phosphorylation and signal activation.

Although the cytoplasmic tails of adaptors are typically depicted as dynamic polypeptide chains whose ITAMs are accessible to protein kinases, this is not the case as there are safety checks in place to prevent ITAM phosphorylation in the absence of receptor engagement (Figure 1). The intracellular chains of adaptor molecules contain basic rich stretch (BRS) sequences composed primarily of positively charged amino acids that interact with negatively charged phospholipids in the inner leaflet of the plasma membrane, thereby anchoring the cytoplasmic tails and sequestering the ITAMs.[9–16] The exact events that are involved at this critical step are incompletely understood; however, we know that recognition of a ligand through its receptor results in proximal changes that release the cytoplasmic tails and render them susceptible to phosphorylation by Src family kinases such as Lck or Fyn (Figure 1).[4,9] Once the tyrosine residues in the ITAMs are phosphorylated, they serve as scaffolds for other kinases including Syk and ZAP70 that initiate a signal transduction cascade that results in cytoskeletal reorganization and activation of PLCγ to induce the Ca^{2+}–calcineurin–NFAT pathway, the NF-κB pathway, and activation of AP-1 transcription factors.[9,17]

Most of our understanding of the proximal events that take place come from studies involving the T cell receptor (TCR) complex; thus,

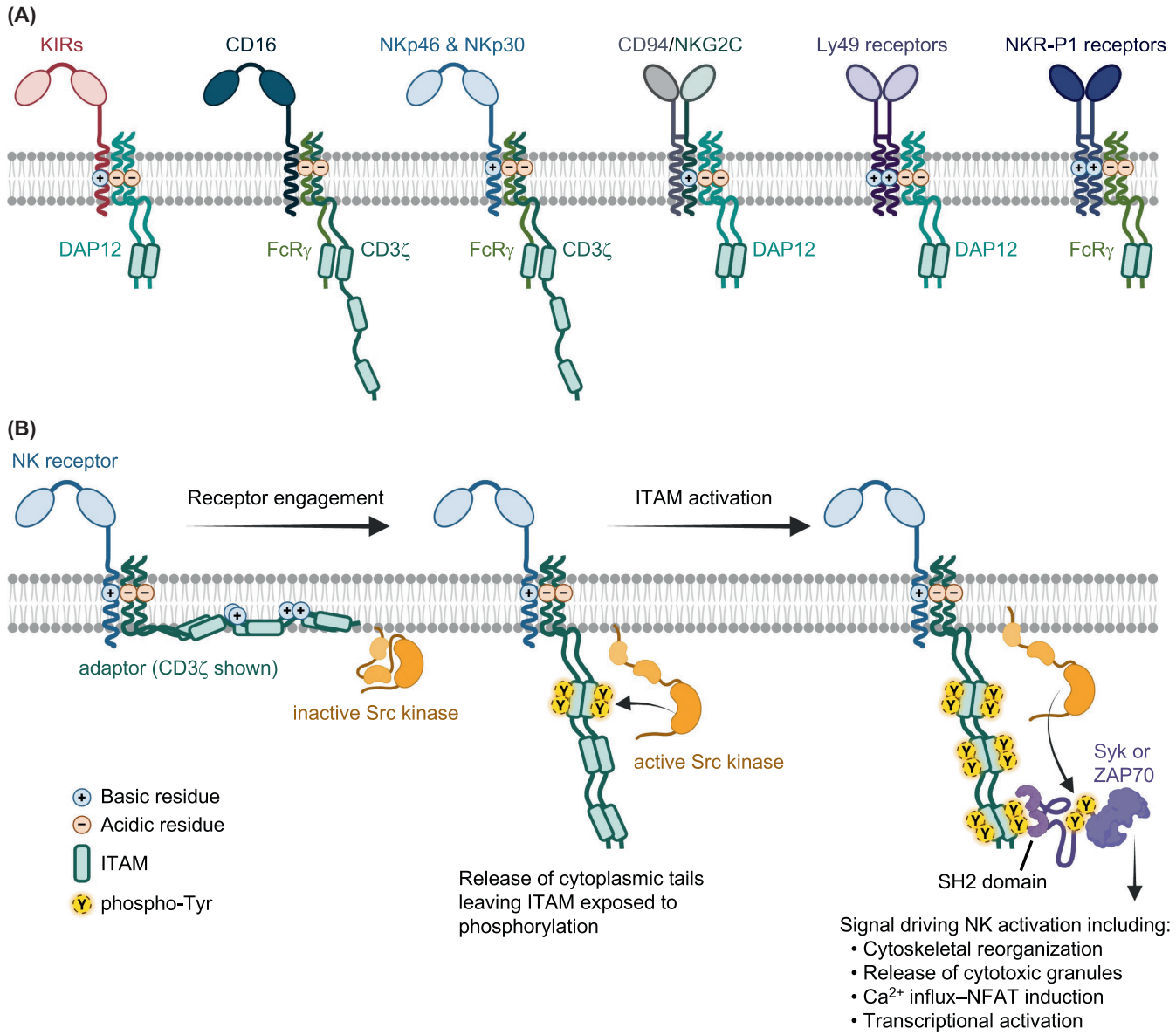

FIGURE 1 ITAM-bearing receptors in natural killer cells. (A) Schematic representation of different receptor complexes highlighting the association of different adaptors with NK receptors. (B) Representation of early events involved in ITAM-mediated NK cell activation. Upon receptor engagement, ITAMs become exposed allowing phosphorylation by Src kinases events. This results in recruitment of Syk or ZAP70 that initiate a signaling cascade indicative of NK cell activation. Figure created with BioRender.com.

understanding how they function in NK cells may offer unique insight. For instance, what dynamic arrangement of the ITAM regions needs to be adopted to make them accessible for phosphorylation? How does cross-linking of multiple receptors change the local concentration of ITAMs, and what effect does this change in concentration have on the kinetics of phosphorylation? Are there different spatial arrangement of ITAMs and how are they differentially accessible to protein kinases? This is important as NK cells express various receptors complexes with different adaptors, each with unique ITAM sequences.[18] As outlined in Table 1, NK cells express a multitude of stimulatory NK receptors that associate with the few adaptors and recognize a vast array or ligands.

3 | ITAM-CONTAINING ADAPTOR MOLECULES

3.1 | CD3ζ and FcεR1γ

The CD3ζ (*CD247*) chain was first identified as being a part of the TCR complex in both mice and humans. Therefore, it is sometimes referred to as TCRζ in addition to CD3ζ (or just ζ chain), although its structure is very different from the other TCR signaling chains. Whereas the other CD3 chains (γ, δ, and ε chains) contain an immunoglobulin (Ig) domain in the extracellular domain and a single ITAM in their intracellular tail, CD3ζ only expresses 9 amino acids extracellularly, contains

TABLE 1 ITAM-associated NK receptors.

Gene	Receptor Name	Adaptor	Species	Ligand	Expression	Reference
FCGR3A	CD16	CD3ζ or FcεR1γ	Human	Fc of IgG	NK, ILC subsets, myeloid cells, T cell subsets	19,20
NCR1	NKp46	CD3ζ or FcεR1γ	Human	Exo-Calreticulin, viral HA, CFP	NK, ILC subsets	21–24
NCR2	NKp44	DAP12	Human	Viral HA, PDGF-DD	NK, ILC subsets	25–28
NCR3	NKp30	CD3ζ or FcεR1γ	Human	B7-H3, HCMV pp65	NK, ILC subsets	27–30
KLRC2/KLRD1	NKG2C/CD94	DAP12	Human	HLA-E: pUL40	NK, T cell subsets	31,32
KIR2DS1	KIR2DS1	DAP12	Human	HLA-C2	NK, T cell subsets	33
KIR2DS2	KIR2DS2	DAP12	Human	HLA-C1, HLA-A*11:01	NK, T cell subsets	34
KIR2DS3	KIR2DS3	DAP12	Human	?	NK, T cell subsets	
KIR2DS4	KIR2DS4	DAP12	Human	HLA-Cw4, HLA-F	NK, T cell subsets	35
KIR2DS5	KIR2DS5	DAP12	Human	HLA-C2	NK, T cell subsets	36
KIR3DS1	KIR3DS1	DAP12	Human	HLA-Bw4, HLA-F, HCMV infection	NK, T cell subsets	33,37–41
KIR2DL4	KIR2DL4	FcεR1γ	Human	HLA-G	NK cells	42–44
Fcgr3	CD16	FcεR1γ	Mouse	Fc of IgG	NK, ILC and T cell subsets, myeloid cells	19,20
Ncr1	NKp46	FcεR1γ	Mouse	Exo-calreticulin, viral HA, CFP	NK, ILC subsets	21–24
Klrb1c	NKR-P1C	FcεR1γ	Mouse	MCMV m12	NK, ILC and T cell subsets	45
Klrb1a	NKR-P1A	FcεR1γ	Mouse	MCMV m12	NK cells	45
Klrb1f	NKR-P1F	FcεR1γ	Mouse	Clr-c, Clr-d, Clr-g	NK, ILCs	46
Klrc2/Klrd1	NKG2C/CD94	DAP12	Mouse	Qa-1^b	NK, T cell subsets	47
Klrc3/Klrd1	NKG2E/CD94	DAP12	Mouse	Qa-1^b	NK, T cell subsets	47
Klra8	Ly49H^{B6}	DAP12	Mouse	MCMV m157	NK cells	48,49
Klra4	Ly49D^{B6}	DAP12	Mouse	H-$2D^d$	NK cells	50
Klra12	Ly49L^{BALB}	DAP12	Mouse	$H2^d$, $H2^f$, or $H2^k$ + MCMV m04	NK cells	51
Klra16	Ly49$P^{Ma/My}$	DAP12	Mouse	$H2^d$ or $H2^k$ + MCMV m04	NK cells	51
Klra18	Ly49R^{129S1}	DAP12	Mouse	?	NK cells	52
Klra21	Ly49$U^{Ma/My}$	DAP12	Mouse	?	NK cells	
Klra23	Ly49W1^{NOD}	DAP12	Mouse	H-$2K^k$, H-$2D^d$, H-2^d or H-2^f or H-2^k + MCMV	NK cells	51

three ITAMs in its intracellular domain, and forms covalently linked dimers through a cysteine in its α-helical TM domain (Figure 2A). In addition, the CD3ζ chain also has an aspartic acid (Asp, D) in its TM domain that is critical for pairing with an arginine (Arg, R) similarly located on associated receptors (Figure 2A). In the case of the TCR complex, these amino acids are highly specific as mutating the aspartic acid to glutamic acid or arginine to lysine results in significant defects in their ability to associate.[53,54] Upon activation, the tyrosine residues in the ITAMs get phosphorylated by Src kinases, thereby rendering them as docking sites for SH2-containing kinases Syk and ZAP-70, initiating ITAM-mediated signaling cascades.

CD3ζ has a closely related protein that similarly forms a dimeric adaptor complex known as FcεR1γ (*FCER1G*, herein referred to as FcRγ). FcRγ was first identified as the γ chain in an Fcε receptor complex, hence its name[55]; however, it is now appreciated that it can associate with a myriad of receptors beyond the Fc receptors (Table 1). Interestingly, the CD3ζ and FcRγ genes are in close proximity to each other on chromosome 1 in both humans and mice, and their similar genomic organization suggests that they likely arose from a gene duplication event.[56] Similar to CD3ζ, the FcRγ adaptor has a very short extracellular portion consisting of 5 amino acids and a TM domain highly conserved with the TM of CD3ζ that includes a cysteine and an aspartic acid (Figure 2A). These two adaptors have such a similar TM structure that, in NK cells, they can form CD3ζ-FcRγ heterodimers in addition to the CD3ζ-CD3ζ and FcRγ-FcRγ homodimers.[8,19,20] The striking difference between these adaptors, however, is that while CD3ζ contains three ITAMs, FcRγ contains a single ITAM.

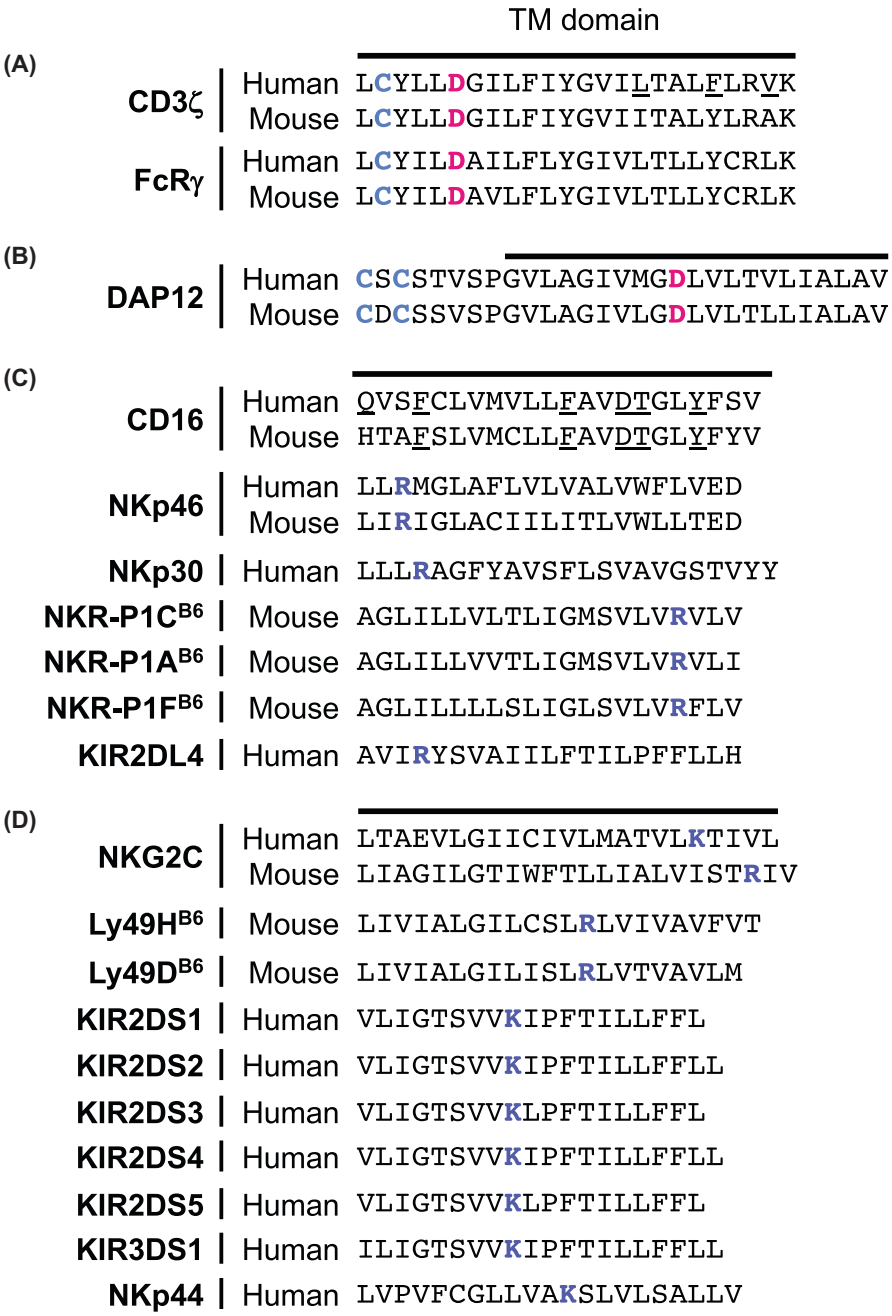

FIGURE 2 Transmembrane domains of adaptor molecules and their associated NK receptors. (A) TM domains of CD3ζ and FcRγ of humans and mice. Residues identified to be important in associating with CD16 are underlined. (B) TM domain of DAP12 in humans and mice. (C) TM domains of CD3ζ- and FcRγ-associated NK receptors in humans and mice. (D) TM domain of DAP12-associated NK receptors in humans and mice. Cysteine residues involved S-S bonds are colored as light blue. Negatively charged amino acids are bolded and colored magenta. Positively charged amino acids are bolded and colored violet. Transmembrane sequences were obtained from UniProt (https://www.uniprot.org/). All sequences are displayed from N-terminal to C-terminal from left to right.

3.2 | DAP12

DAP12 is an ITAM-containing signaling subunit that can associate with several receptors expressed by both human and mouse NK cells, as well as other cell types.[34] DAP12 is a disulfide-linked homodimeric protein that was initially discovered in a human dendritic cell library and has since been identified across many immune cell types including myeloid cells, B cells, T cells, and NK cells.[3] Like the CD3ζ and FcRγ adaptors, DAP12 has a minimal extracellular region with no ligand binding capabilities and a single-pass TM domain with an aspartic acid within the alpha helix (Figure 2B). Each monomer in the DAP12 homodimer contains a single intracellular ITAM that enables phosphorylation and downstream signaling. The acidic residue (aspartic acid) on each of the monomers enables stable, noncovalent association with the single basic residue (lysine or arginine) of a ligand-binding receptor subunit in the non-polar membrane. In NK cells, DAP12 is capable of associating with a diverse set of receptors in humans and mice (Table 1, Figure 2).

4 | NATURAL KILLER CELL RECEPTORS ASSOCIATED WITH ITAM-BEARING ADAPTORS

4.1 | Receptors associated with CD3ζ and FcεR1γ

In line with the similarities between CD3ζ and FcRγ, in human NK cells, these adaptor molecules also share the receptors they form complexes with. One of these receptors is the CD16 (CD16A, FcγRIIIA, *FCGR3A*) Fc receptor for IgG. The CD16 receptor is highly expressed in human NK cells and is a marker of maturity in peripheral blood NK cells. This receptor gives NK cells the ability to exploit the antigen specificity of antibodies by engaging the Fc region of IgG on antibody-coated cells, thereby eliciting antibody-dependent cellular cytotoxicity (ADCC). The CD16 receptor binds IgG with relatively low affinity, thereby only triggering activation when engaged in a multimeric complex, such as on a cellular surface. In humans, CD16 is the sole receptor capable of potently activating resting NK cells.[57] Alleles of CD16 have also been identified that have varying affinity for IgG, including a variant with a phenylalanine to valine substitution at amino acid position 176 (sometimes referred to position 158) yielding higher affinity for IgG.[58] In addition, afucosylated antibodies are more potent at triggering CD16, a feature that is often observed with antibodies to enveloped viruses.[59] Structurally, CD16 contains two extracellular immunoglobulin domains involved in recognition of IgG-Fc, a single-pass TM domain, and a short intracellular tail. Interestingly, the CD16 receptor lacks a positively charged residue in its TM domain and therefore relies on careful packing of amino acids on adaptors to accommodate its TM domain.[8,60] Although in vitro human CD16 can recruit CD3ζ and FcRγ equally,[8] whether there is a preference by CD16 for a specific adaptor complex (homo- or hetero-dimer) in NK cells, either due to structural stability or due to adaptor abundance remains to be determined. Interestingly, in addition to the adaptor molecules, the short intracellular tail of CD16 is also important for signaling as truncation significantly dampens activating signals.[61]

In addition to CD16, the CD3ζ and FcRγ adaptor molecules also form complexes with two of the Natural Cytotoxicity Receptors (NCR). Humans express three NCRs, but only NKp46 (*NCR1*) and NKp30 (*NCR3*) associate with CD3ζ and FcRγ. Of these, only the NKp46 receptor has a homolog in mice while NKp30 is a pseudogene, and no gene has been identified for NKp44.[62,63] Unlike NKp46 and NKp30, NKp44 (*NCR2*) associates with DAP12 (see below).[64] NKp46 contains two extracellular immunoglobulin domains while NKp30 and NKp44 have a single Ig domain followed by a single-pass TM domain and a short cytoplasmic tail. NKp46 and NKp30 contain an arginine residue in the α-helices of their TM domains. Arginine plays a critical role in assembly with either CD3ζ or FcRγ through interaction with the acidic residue, aspartic acid (Figure 2A, Figure 2C). These receptors bind a diverse array of ligands. NKp46 was first reported to bind viral hemagglutinin and hemagglutinin neuraminidases[21,65,66] and has since been reported to bind to heparin sulfate proteoglycans,[22] and complement factor P.[23] Recently, it was shown that NKp46 can also detect externalized calreticulin, which accumulates on the surface of cells undergoing ER stress.[24] NKp30 was first shown to bind B7-H6,[29] a type I transmembrane protein that contains two Ig domains and is a ligand often expressed on tumor cells but not healthy cells.

Mice also express activating NKR-P1 receptors. These genes are encoded centromerically to the NKG2 family and the Ly49 family of NK receptors in the Natural Killer Gene Complex (NKC). Like most members of NK receptors in the NKC, the NKR-P1 receptors are type II transmembrane proteins with extracellular C-type lectin-like domains. This family of receptors contains five members, two which are inhibitory (NKR-P1B/*Klrb1b* and NKR-P1G/*Klrb1*) and three stimulatory receptors (NKR-P1C/*Klrb1c*, NKR-P1A/*Klrb1a*, and NKR-P1F/*Klrb1f*).[67,68] The NKR-P1C receptor is also known as NK1.1 in C57BL/6 (B6) mice as it is the receptor that is recognized by the PK136 monoclonal antibody.[69–71] NKR-P1C contains a positively charged arginine residue in its TM domain. Interestingly, NKR-P1C associates with FcRγ but not with homodimers of CD3ζ or heterodimers of CD3ζ-FcRγ.[72] Consequently, NKR-P1C requires FcRγ for signaling[72,73]; however, it does not require an adaptor for cell surface expression like most other multicomponent receptors.[72] This could be explained by the fact that NKR-P1C forms homodimers that may stabilize charged amino acids in the TM domain of the receptor despite having these residues exposed. While it has not been confirmed that NKR-P1A and NKR-P1F associate with FcRγ, this is highly likely given the high similarity of their TM domains to that of NKR-P1C (Figure 2C). The ligand for NKR-P1C is the mouse cytomegalovirus (MCMV)-encoded glycoprotein m12, which also interacts with an inhibitory member of the NKR-P1 family, NKR-P1B.[45] Through mechanisms that are still unknown, despite having similar affinities for NKR-P1C and NKR-P1B, m12 preferentially interacts with the inhibitory NKR-P1B receptor.[45] NKR-P1A also interacts with m12 although the affinity between these molecules

is unknown, and this receptor is weakly detected on surface of NK cells in B6 mice.[74] The high degree of polymorphisms found across NKR-P1A, NKR-P1B, and NKR-P1C suggests that these receptors are under pressure from pathogen-driven evolution, and this was confirmed through the identification of the m12 viral ligand that is engaged by all of these receptors, to different degree in different mouse strains.[16,45,67] NKR-P1F interacts with genetically linked C-type lectin-like proteins Clr-c, Clr-d, and Clr-g. The importance of these interactions remains to be determined. Humans express a single ortholog of the NKR-P1 family, the inhibitory NKR-P1A, also known as CD161, that interacts with a genetically linked C-type lectin-like protein LLT-1 (also known as *CLEC2D*).[75,76] Interestingly, there are other genetically linked C-type lectin-like receptors and ligands (NKp80:AICL and NKp65:KACL) in the human NKC encoded by genes close to NKR-P1A and LLT-1; however, it remains unclear if these are homologous genes.[77] However, the NKp65 and NKp80 do not associate with adaptor molecules and instead signal through hemITAM sequences.[78,79]

4.2 | Receptors associated with DAP12

The NKG2/CD94 family of NK receptors are type II transmembrane proteins that form heterodimers with the invariant CD94 subunit. These receptors contain C-type lectin-like extracellular domains that are involved in ligand recognition and include inhibitory members (NKG2A or B) and in humans an activating member, NKG2C.[31,47,80] These receptors recognize MHC class Ib molecules (HLA-E in humans and Qa-1^b in mice), which contain a peptide groove that accommodates leader peptides of other MHC class I molecules.[31] The activating human NKG2C/CD94 forms a receptor complex through charged interactions between a lysine in the TM of NKG2C with the aspartic acid pair on DAP12.[7,31,81] Interestingly, although the mouse NKC2C/CD94 receptor similarly forms a complex with DAP12, and despite the presence of an arginine in the TM of NKG2C, association with DAP12 involves a lysine residue in the TM of CD94.[82]

The Killer cell Immunoglobulin-like Receptor (KIR) family represents the largest family of NK receptors in humans. These receptors can either contain 2 or 3 immunoglobulin-like domains, a single-pass TM domain, and a varying length of intracellular chain. Like most families of NK receptors, the KIRs consist of both activating and inhibitory members that can be identified by the length of the cytoplasmic tails as being short (S) or long (L), respectively. The inhibitory KIRs contain a long cytoplasmic tail that encodes an immunoreceptor tyrosine-based inhibitory motif (ITIM),[2] while the activating KIRs assemble with DAP12 through intermolecular interactions involving a lysine situated centrally in their TM domain and aspartic acid residues on DAP12 (Figure 2B). Although the KIRs have highly polymorphic Ig-like extracellular domains, the TM domain is extremely well conserved (Figure 2D). Many of these receptors recognize highly polymorphic class I human leukocyte antigen (HLA) genes, and consequently, the KIR genes similarly exhibit remarkable variability.[83,84] Crystal structures have revealed that the KIR docks with class I HLA proteins near the residue at position 8 of the peptide loaded into the HLA groove.[85] In agreement with the structural analysis, a recent study revealed that activating KIR has high specificity for peptides at position 7 and 8.[86] Thus, it is likely that peptides presented during pathogenesis drive activation through activating KIRs; however, this remains to be determined physiologically.

Rather than relying on KIRs for recognition of self-MHC class I molecules, mice expanded the Ly49 family to serve this function. Like the NKG2 and NKR-P1 receptors, the Ly49 receptors are type II transmembrane proteins with extracellular C-type lectin-like domains. Akin to the KIRs, the Ly49 receptors are highly polymorphic and consist of both inhibitory and stimulatory members based on the presence of an ITIM in their intracellular tail or a charged arginine or lysine in their TM domain, respectively. The inhibitory receptors of this family interact with MHC class I molecules.[87] These receptors are also targeted by viral immunoevasive proteins.[48,88,89] The most compelling evidence involves a mouse cytomegalovirus (MCMV)-encoded cell surface protein, m157. This protein likely evolved to inhibit NK cells through engagement with Ly49I and Ly49C at an affinity higher than interactions with the self-H-2 ligands.[48,89] This mechanism is highly effective at dampening NK cell responses in several mouse strains except C57BL/6 mice. B6 mice encode the Ly49H receptor that associates with DAP12 and effectively recognizes m157 to control viral infection.[48,49,90] It is likely that the Ly49H receptor evolved as a result of a recombination event involving the exons that encoded a Ly49I extracellular domain with those that encoded the TM domain and intracellular domain of an activating receptor such as Ly49D.[67] Recognition of MCMV infection has also been documented by other activating Ly49 receptors in different mouse strains ($Ly49L^{BALB}$, $Ly49P^{Ma/My}$, $Ly49W1^{NOD}$; Table 1).[51]

Humans also contain two receptors that do not fit the typical profile. These are the KIR2DL4 and NKp44 receptors. Both receptors contain extracellular Ig domains, a single-pass TM domain, long cytoplasmic tails that contains ITIMs, and a charged residue in their transmembrane that facilitates association with adaptor molecules.[25,42] NKp44 associates with DAP12 while KIR2DL4 associates with FcRγ. This is in line with what would be expected based on the position of the charged residues within the TM domains (FcRγ has an aspartic acid closer to the surface while DAP12's aspartic acid is located more centrally in the TM domain; Figure 2). NKp44 has been reported to bind viral hemagglutinin and the platelet-derived growth factor (PDGF)-DD.[26] KIR2DL4 has been reported to interact with the MHC class Ib molecule HLA-G.[91] Due to their ability to drive both activating and inhibitory signals, it has been challenging to pinpoint the function of these receptors.

5 | DIFFERENCES BETWEEN HUMAN AND MOUSE

The common ancestor between humans and mice can be traced back to the late Cretaceous period, about 90 million years ago.[92] Therefore, it is not surprising that the evolutionary pressures throughout these

millions of years caused significant diversion at the genetic level. In fact, only about 80% of the amino acid sequences are conserved between humans and mice with about 14,000 out of about 20,000 genes in both species sharing a direct common ancestor. Many of these differences have been well documented for various aspects of the immune system.[93] This is not factoring the differences in microbiota between humans and mice, and in particular, laboratory mice.[94]

These differences are abundantly clear when it comes to comparisons of human and mouse NK cells. Firstly, NK cells in the two species are identified using different markers. In humans, NK cells are generally identified as being $CD56^{+}CD3^{-}CD14^{-}$ lymphocytes, and within this gate are the two main NK cell subsets, the immature $CD56^{bright}CD16^{-}$ and the mature $CD56^{dim}CD16^{+}$ NK cells. In contrast, NK cells in mice do not express CD56 and instead are identified as being $CD49b^{+}NKp46^{+}CD3^{-}$ (universally across mouse strains). Human and mouse NK cells both express NKp46; however, NK cells in humans have low expression, making it a difficult marker to use for identification. In B6 mice, all NK cells express the NKR-P1C (NK1.1) receptor, which is recognized by the PK136 monoclonal antibody. Although PK136 stains all NK cells (and some ILCs and some minor T cell subsets) in B6 mice, this is not always the case across different mouse strains. In some strains that stain positive for NK1.1, the PK136 antibody is not reacting with the same receptor, and thus only a subset of cells are detected, as is the case with SJL and FVB/Swiss strains where PK136 stains NKR-P1B.[67–69] Secondly, the evolutionary pressure on many NK receptors is so much that they do not share many receptor families. In humans, the predominant receptors involved in recognition of self-MHC are the inhibitory KIR. In mice, this function is mediated by the inhibitory Ly49 receptors. Many of the activating receptors in both receptor families have also evolved to recognize non-self ligands (Table 1). In fact, of the ITAM-based NK receptors, NKp46 is the only receptor shared between humans and mice and shares 61.18% amino acid similarity.

Although both humans and mice express Fc receptors with shared expression and function, the genes encoding these two receptors, CD16A (*FCGR3A*) in humans and CD16 in mice (*Fcgr3*), do not share the same gene homolog. The human CD16A shares ancestry with CD16.2 (*Fcgr4*), which is not expressed on mouse NK cells, while the mouse CD16 receptor shares ancestry with the CD32A (*FCGR2A*) and CD32C (*FCGR2C*) receptors in humans.[95–97] In addition, we recently highlighted key differences in the function of the FcγR complexes between these species.[60,98] Whereas human NK cells are efficiently activated through their Fc receptor, NK cells from mice are weakly activated. This is due to polymorphisms in three amino acids in the TM domain of CD3ζ such that the human CD16A can associate with human CD3ζ (in addition to FcRγ), whereas mouse CD16 cannot associate with mouse CD3ζ and instead relies on FcRγ alone[60] (Figure 2A).

The impact of these polymorphisms in the TM domain of mouse CD3ζ is also seen in other receptors beyond CD16. Analysis of mice lacking FcRγ reveals that in addition to not being able to express CD16, NK cells from *Fcer1g*$^{-/-}$ mice also lack expression of NKp46 (Figure 3A).[15,99,100] In addition, these NK cells are not capable of mounting responses upon cross-linking with NK1.1, NKp46, or CD16 receptor-specific antibodies (Figure 3B). Therefore, these results reveal that in mouse NK cells, NK1.1, NKp46, and CD16 require FcRγ, whereas CD3ζ is dispensable.[100] A puzzling observation is that while NK cells in mice are capable of being activated through NK1.1 and NKp46, CD16 only weakly activates NK cells. Why only the CD16 receptor is negatively affected is unknown, although elements in the TM domains are likely suspects as both NK1.1 and NKp46 have an arginine (R) in their TM domain while CD16 lacks a positively charged residue in its TM domain (Figure 2C). Nevertheless, this does not explain why these receptors cannot signal through CD3ζ.

To determine if human CD3ζ could associate with these FcRγ-associated mouse NK receptors, we transduced the human NK cell line, NK-92, with retroviruses expressing these receptors and assessed the functionality of the receptor complexes. We have previously shown that NK-92 transduced with mouse CD16 form functional receptors using human CD3ζ.[60] We similarly transduced NK-92 with either mouse NKp46 or mouse NK1.1 ($NKR\text{-}P1C^{B6}$) and determined their function. NK-92 cells could express mNKp46 and mNK1.1 on their cell surface (Figure 4A). However, when we performed plate-bound stimulation assays using antibodies to these receptors, mouse NKp46 was potently capable of activating NK cells, whereas NK1.1 was very weakly responsive (Figure 4B). Therefore, these results demonstrate that the TM domain of CD3ζ is also critical for its ability to associate with NKp46 in both human and mouse. NKR-P1C, on the other hand, can make it to the cell surface independent of adaptor molecules (Figure 3A, Figure 4A) and only weakly associates with CD3ζ (Figure 4B). Given that FcRγ and CD3ζ have such high conservation of their TM domains, understanding the mechanisms responsible for these differences would be important in understanding NK cell receptor assembly and function.

6 | NK CELL MEMORY

Natural killer cells had long been considered to be short-lived cells that are constantly replenished throughout an individual's life. This dogma was challenged by several studies demonstrating that NK cells can "remember". This memory response was first shown by the von Andrian laboratory, which, using a model of hapten-induced contact hypersensitivity, reported that *Rag2*$^{-/-}$ mice were able to mount a recall response.[101,102] This observation was mapped to a population of hepatic $CXCR6^{+}$ innate lymphoid cells, which, upon retrospective analysis are likely ILC1s.[101,102] However, the receptors that drive this mechanism are unknown. Recent work suggests that inhibitory receptors may be involved.[103,104] In addition, the Yokoyama lab demonstrated that NK cells primed with IL-12, IL-18, and IL-15 were also able to mount enhanced secondary responses and lived longer.[105] However, the strongest evidence for a role of antigen specificity in the generation of memory NK cells comes from individuals with cytomegalovirus (CMV) infection, a feature that has been observed in both humans and mice. This was elegantly described by the Lanier lab when Joseph Sun made the seminal observation in B6 mice infected with MCMV.[106] Upon infection, NK cells recognize virally infected cells through their Ly49H

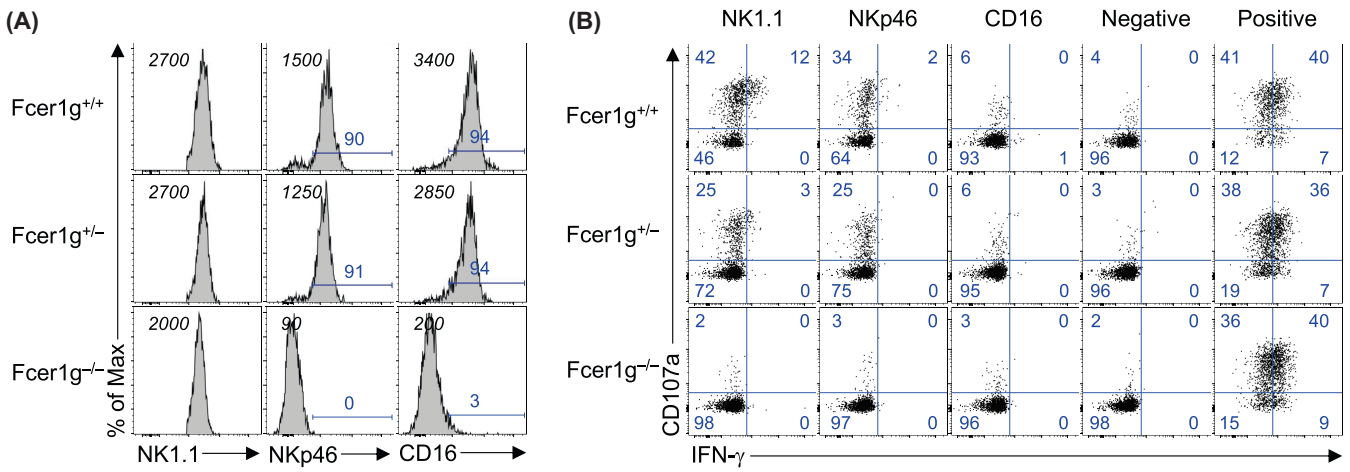

FIGURE 3 *Fcer1g*-deficient NK cells cannot be activated through NK1.1, NKp46, or CD16. Splenocytes were harvested from *Fcer1g*$^{+/+}$ (WT), *Fcer1g*$^{+/-}$ (Het), and *Fcer1g*$^{-/-}$ (KO) mice and (A) analyzed for cell surface expression of NK receptors, and (B) stimulated with antibodies against activating receptors or controls in plate-bound stimulation assays measuring CD107a (degranulation) and IFN-γ expression (cytokine secretion) on NK cells (CD49b^{+}CD3^{-}NK1.1^{+}) using flow cytometry. In (A), numbers of top left corner of plot show the median fluorescence intensity (MFI) whereas the numbers in blue represent % of marker^{+} NK cells. In (B), numbers in blue represent % of NK cells that are found in each of the gates. PMA + ionomycin was used as a positive control.

activating receptor that engages the MCMV-encoded protein m157. Receptor engagement results in the proliferation of Ly49H^{+} NK cells to control the virus,[107,108] and a subset of these cells persist to become long-lived memory NK cells with enhanced protection to re-challenge with MCMV.[106,109] Subsequently, it was shown in humans that certain individuals seropositive for HCMV had an expansion of NKG2C^{+} NK cells.[110–112] In recent years, a unique subset of CD56^{dim}CD16^{+}NKG2C^{+} NK cells has been described that undergo epigenetic reprogramming, frequently including loss of FcRγ and Syk,[113,114] and are more effective at killing antibody-coated cells.

7 | NK CELL RECEPTORS INVOLVED IN GENERATING MEMORY

7.1 | Adaptive NK cells in humans

The initial discovery of memory NK cells in mice infected with MCMV prompted a more careful analysis of the NK receptor repertoire in humans seropositive for human cytomegalovirus (HCMV). Previous studies had highlighted imprinting of HCMV on the NK repertoire after infection.[111,115] Subsequent studies revealed that some, but not all, individuals seropositive for CMV have an expansion of NK cells positive for NKG2C and with higher receptor density,[110–112] akin to the observation of Ly49H expression on NK cells in B6 mice infected with MCMV. This suggested that the NKG2C/CD94 receptor could be engaging a HCMV-induced complex. In agreement with this hypothesis, it was discovered that the leader peptide from the HCMV-encoded protein UL40 is capable of being loaded into the peptide binding groove of HLA-E and results in cell surface expression (HLA-E:pUL40) to inhibit NK cells by engaging the NKG2A/CD94. However, some individuals likely evolved the ability to circumvent this inhibition by using the NKG2C/CD94 receptor to recognize this HLA-E:pUL40 complex.[116–118] The recognition of HLA-E:pUL40 by NKG2C/CD94 NK receptor is influenced by the amino acid sequence of the UL40 leader peptide as different HCMV isolates display polymorphisms that differently engage the receptor and result in different levels of NK cell activation and expansion of NKG2C^{+} cells.[32,119] Although it is challenging to prove in human that HCMV is what drives the generation of NKG2C^{+} adaptive NK cells, studies from non-human primates have demonstrated the ability of CMV to generate adaptive NK cells after experimental CMV infection.[120] In humans, these NKG2C^{+} NK cells undergo epigenetic programming that often results in the loss of the FcRγ adaptor molecule, along with other signaling molecules including Syk.[113,114] Interestingly, these FcRγ^{-} NK cells have enhanced CD16 responses,[121] likely due to the reliance on CD3ζ that is capable of driving stronger signals due to increased ITAMs. This hypothesis was recently supported through CRISPR-mediated editing of *FCER1G*.[121] Additionally, during active HCMV reactivation in renal transplant patients, it was shown that NKG2C^{+} NK cells proliferate; however, these NKG2C^{+} cells do not expand from the adaptive NK cell pool. Rather, there is a subset of NKG2C^{+}FcRγ^{+} pre-memory-like NK cells that proliferate and give rise to NKG2C^{+}FcRγ^{-} adaptive NK cells.[122] This finding is consistent with the observation that the pool of adaptive NK cells comes from clonal bursts that give rise to long-lasting cells and not from a single source that continually expands.[123] Further, NKG2C^{+}FcRγ^{-} NK cells are poorly proliferative in vitro.[124] These observations demonstrate that NKG2C/CD94 is important for driving the generation of adaptive NK cells in humans and non-human primates. Importantly, this process involves signaling cascades through the DAP12 adaptor molecule.

7.2 | Adaptive NK cells in mice

The mouse model has provided much more insight into the molecular underpinnings that drive the formation of adaptive NK cells. The

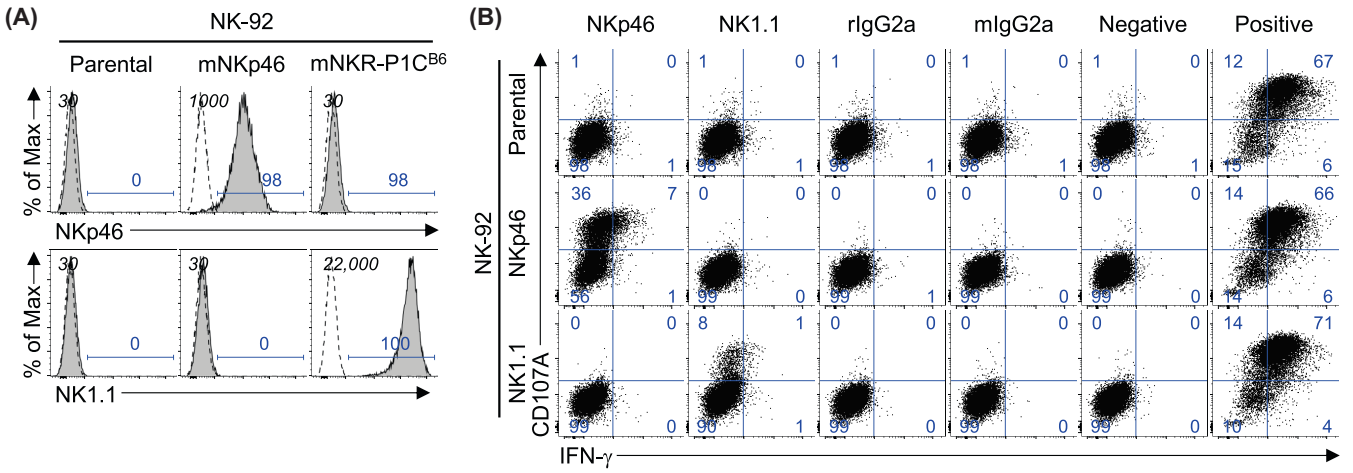

FIGURE 4 Human CD3ζ can associate with mouse NKp46 but not mouse NKR-P1C^{B6}/NK1.1. The human NK cell line NK-92 was transduced with retroviral vector expressing mouse NKp46 or mouse NKR-P1C^{B6}/NK1.1. (A) After sorting, cells were analyzed for cell surface expression of intended markers using flow cytometry. Numbers of top left corner of plot show the MFI whereas the numbers in blue represent % of marker^{+} NK-92 cells. (B) NK-92 cells were activated using antibodies against mouse NKp46 (29A1.4), NK1.1 (PK136), or controls, in plate-bound antibody stimulation assays. Numbers in blue represent % of NK-92 cells that are found in each of the gates. PMA + ionomycin was used as a positive control.

generation of adaptive NK cells during MCMV infection is dependent of antigen recognition through Ly49H.[106] However, Ly49H alone cannot drive adaptive NK cell formation as this Ly49H-mediated signal requires synergy with type I interferons,[125] IL-12, and IL-18 for expansion,[126,127] and IL-15 for maintenance.[128] Adaptive Ly49H^{+} NK cells also undergo epigenetic remodeling, although interestingly and in contrast to humans *Syk* or *Fcer1g* are not affected.[129]

Several studies have investigated the role for other NK receptors in driving this differentiation. Adaptive NK cell formation requires the co-stimulatory molecule DNAM-1,[130] but not the NKG2D receptor.[131] The NKG2D receptor augments NK cell expansion during infection, but it alone cannot drive memory NK cell formation.[131] Interestingly, DAP10 can also form complexes with Ly49H that support the expansion during infection, but whether these signals are important to drive memory formation remain to be determined.[132] With respect to inhibitory receptors, the majority of Ly49H^{+} NK cells that expand during infection are Ly49I^{-},[108,109] and NKR-P1B^{-},[133] highlighting that inhibitory receptors dampen the Ly49H-mediated expansion. Studies in mice with CD16-deficient NK cells revealed no involvement for CD16 in mice during infection.[98] However, studies in C57BL/6 mice expressing H-2D^{d} (the ligand for the stimulatory Ly49D receptor) found that Ly49H synergized with Ly49D to enhance NK cell activation and generation of memory.[134] Thus, it is likely that other NK receptors may similarly support the transition of conventional to adaptive NK cells.

Despite all the differences between the two species, the examples of adaptive NK cells in humans and mice have one important feature in common: both NKG2C/CD94 in humans and Ly49H in mice signal through the DAP12 adaptor molecule. Therefore, is DAP12 the only receptor that can drive this differentiation? Or can other receptors, through other adaptors also yield adaptive NK cells? In vitro stimulation of human NK cells upon stimulation of their CD16 receptor can drive enhanced secondary responses[135]; therefore, there may be other ways to drive this program in NK cells. It has been shown that avidity of receptor density at the cell surface drives preferential expansion[136,137]; however, the mechanisms that are responsible for these differences and whether adaptor molecules play a role here remain to be determined. There is a case for synergy involving Ly49H with other NK receptors; however, not all strains of MCMV encode m157; therefore, are there scenarios where other receptors drive a similar response? For instance, can recognition of m12 by the activating NKR-P1C/NK1.1 receptor drive a memory response in NK cells?[45,138] Of course, a central question is, is this response specific to CMV? Or can other viruses promote similar responses? If not specific to CMV, What are the other receptors involved in generating adaptive NK cells?

8 | CONCLUDING REMARKS

The last five decades investigating NK cells and how they are activated have provided much insight about the receptors and ligands that they recognize. Recently, we have begun to appreciate how activation through these receptors give rise to specialized NK cell subsets with adaptive features that have blurred the line between adaptive and innate lymphocytes. However, it is evident that we need to have a better understanding of what makes receptors assemble efficiently, how these receptors are arranged to synergize, what are the signaling cascades that are driven by the different receptors and how these influence the effector functions, maturation, and ability of NK cells to 'remember'. It is abundantly clear that NK cells offer a multitude of features that make them attractive cellular therapeutics, but to maximize their potential, we must address the questions presented here among others. In particular, understanding ITAM-mediated signals is instrumental to this goal.

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DISCLOSURE

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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