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Review

Evolution of the Ly49 and Nkrp1 recognition systems

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ABSTRACT

The *Ly49* and *Nkrp1* loci encode structurally and functionally related cell surface proteins that positively or negatively regulate natural killer (NK) cell-mediated cytotoxicity and cytokine production. Yet despite their clear relatedness and genetic linkage within the NK gene complex (NKC), these two multi-gene families have adopted dissimilar evolutionary strategies. The *Ly49* genes are extremely polymorphic and evolutionarily dynamic, with distinct gene numbers, remarkable allelic diversity, and varying MHC-I-ligand specificities and affinities among different murine haplotypes. In contrast, the *Nkrp1* genes have opted for overall conservation of genomic organization, sequences, and ligand specificities, with only limited and focused allelic polymorphism. Possible selection pressures driving such varied evolution of the two gene families may include disequilibrium from ligand co-inheritance, pathogen immunoevasin strategies, flexibility in host counter-evolution mechanisms, and the prevalence and dynamics of inherent repetitive elements.

1. Introduction

Within a given species, different individuals do not always share the same complement or number of genes. In fact, it is becoming increasingly apparent that an individual's distinctiveness derives in part from subtle genomic nuances, each a product of unique combinations and permutations of gene sets that are strikingly polymorphic and polygenic. While this intraspecies genomic diversity has been known to exist for some time amongst the genes encoding the odorant receptors (OR) and the major histocompatibility complex (MHC) proteins [1,2], more recently it has been shown to hold true for the genes encoding natural killer cell receptors (NKR) specific for class I MHC; namely, the murine *Ly49* genes and their human analogs, the killer cell immunoglobulin-like receptors *(KIR)* genes. Notably, all three gene families (OR, MHC, NKR) are components of sensory systems, responsible for distinguishing self from nonself, or normal from "danger". However, unlike many innate pattern recognition (PR) systems, which have also evolved to sense nonself or danger cues [3], the specificities of the Ly49 and KIR systems are not "hardcoded" into the genome or conserved between individuals. Indeed, a number of striking features sets these NK recognition systems apart from typical PR genes, including their remarkably diverse, polymorphic, polygenic, modular, and adaptable nature, as well as their organizational plasticity and restricted co-localization within a single compartmentalized region of the genome (the NKC [4] or LRC [5], respectively). Indeed, it appears that clustering of the *Ly49/KIR* genes likely promotes their rapid duplication and deletion, resulting in altered specificities as well as nonfunctional pseudogenes; in contrast, the relative isolation and segregation strategy employed by typical PR genes may result in enhanced conservation and a 'hardwiring' of specificities. In support of this generalization, consider the interesting exception of *Ly49b*, a single isolated gene conserved among rodent strains yet located outside the main *Ly49* gene cluster; this segregated gene appears to encode a relatively invariant pan-MHC-specific receptor on macrophages [6,7].

On the other hand, unlike the majority of *Ly49* family members, the structurally and functionally related *Nkrp1* gene family appears to possess a highly stable genomic organization and displays only limited polymorphism within a given species [8–10]. While the functions of these two NKR gene families are similar in many ways, the *Ly49* system employs a dynamic, polymorphic, and non-isogenic approach, while the *Nkrp1* system has opted for a stable genetic organization and only minimal (oligomorphic) diversity that may be indicative of resistance to evolutionary diversification. In this review, we attempt to elucidate possible reasons behind

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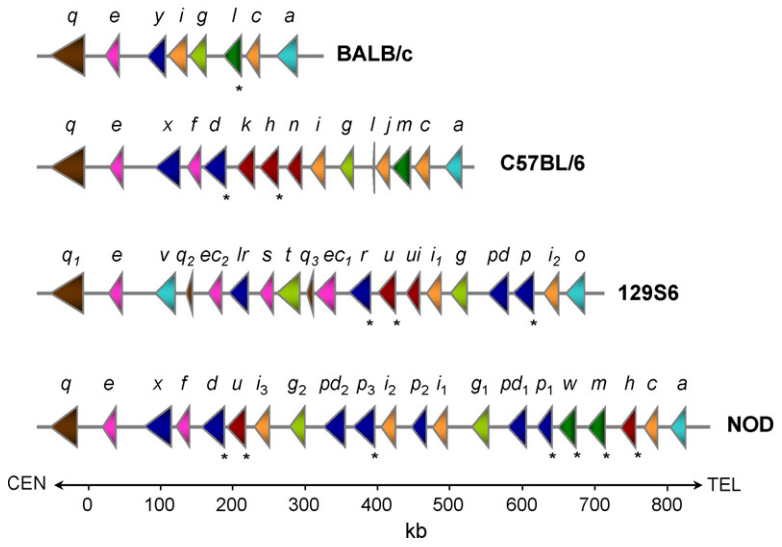

Gene #	S:I:Ψ Ratio
8	1:6:1
15	2:8:5
19	3:9:7
21	7:6:8

Fig. 1. Comparison of gene content between four divergent murine *Ly49* haplotypes. The schematic organization of the *Ly49* gene cluster is depicted for four inbred mouse strains (BALB/c, C57BL/6, 129S6, NOD), along with a summary of gene numbers and receptor isoform usage (S:I:ψ ratio, stimulatory versus inhibitory isoform versus pseudogene ratio). The orientation and relative span of the genes are indicated by the direction and size of the triangles (scalebar below in kilobases). Different gene colors represent distinct *Ly49* subfamily relationships. Genes encoding stimulatory Ly49 receptors are denoted with an asterisk (*).

the different evolutionary strategies utilized by the *Ly49* and *Nkrp1* recognition systems.

2. Genomic organization: opposing evolutionary pathways within the NKC

What promotes NKR gene polymorphism, polygenicity, and non-isogenic inheritance? One likely candidate is pathogen-driven selection. As deficient NK cell responses can lead to debilitating viral infections [11], the complement of NKR inherited by an individual may crucially influence disease resolution. Indeed, the presence or absence of NKR within distinct haplotypes of a given species can directly impact host resistance or susceptibility during the course of infection. In turn then, what genetic basis and/or molecular mechanisms are behind this variability and why is immune resistance not 'hardcoded' into the genome if it confers a significant advantage or disadvantage to survival? While there are no simple answers to these questions, it is likely that strain-specific diversity evolved in part under pressure from multiple distinct pathogens affecting distinct host populations, each attempting to maintain an NKR repertoire that is both useful and self-tolerant. Here, we explore the known *Ly49* repertoires of common mouse strains to shed light on possible mechanisms of expansion and contraction.

2.1. The Ly49 system: polymorphic receptors for segregating polymorphic MHC-I ligands

After the cloning of the first Ly49 cDNAs [12,13], investigations into genomic restriction fragment length polymorphisms indicated that the *Ly49* designation likely encompassed a multigene family maintaining considerable variability [14]. With the subsequent mapping and complete sequencing of four murine *Ly49* haplotypes (Fig. 1), as well as multiple human *KIR* clusters [15], it is now obvious that the MHC-I-specific NKR repertoires are remarkably variable in gene content [16–19]. On the other hand, these NKR clusters also possess limited conservation, including 'framework' genes that are maintained in all of the known mouse *Ly49* or human *KIR* haplotypes. These conserved genes provide a scaffold within which regions of variable numbers of unique strain-specific genes reside. In mice, there are three pairs of framework genes (*Ly49q-e, Ly49i-g*, and *Ly49c-a*) [18] (Fig. 2). While beyond the scope of this review, the human *KIR* haplotypes appear to be similarly complex and also contain framework genes [15,20]. Interspersed between these anchor positions in the genome, there are known to exist up to a total of 8–21 genes in distinct mouse *Ly49* haplotypes. The following is a summary of the major characteristics of the four sequenced murine *Ly49* haplotypes:

2.2. B6

C57BL/6 was the first *Ly49* haplotype to be characterized, initially by individual laboratories, then subsequently by the mouse genome sequencing initiative [21–23]. B6 mice possess a *Ly49* cluster containing 15 identifiable genes (Fig. 1). Of these, only two encode functional stimulatory receptors (*Ly49d, h*), while eight encode functional inhibitory receptors (*Ly49q, e, f, i, g, j, c, a*). The remaining genes are pseudogenes and do not code for functional proteins. Relevant to pathogen recognition, the Ly49H stimulatory receptor directly interacts with the MCMV-encoded MHC-like [24] m157 gene product on infected cells [25,26]. This interaction provides a molecular basis for the notable resistance of B6 mice to MCMV infection. As detailed below, the m157 immunoevasin has also been shown to interact with the inhibitory $Ly49I_1^{129}$ receptor, which it presumably evolved to target using a "decoy" ligand strategy. The B6 haplotype is also shared with C56BL/10 mice.

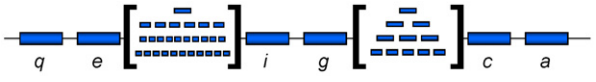

Fig. 2. Framework model of a minimal mouse *Ly49* haplotype. Framework *Ly49* gene pairs conserved among all mouse strains are depicted by large boxes and labeled, while variable numbers of non-conserved *Ly49* genes, which distinguish each haplotype, are enclosed in brackets in the intervening regions of the genome.

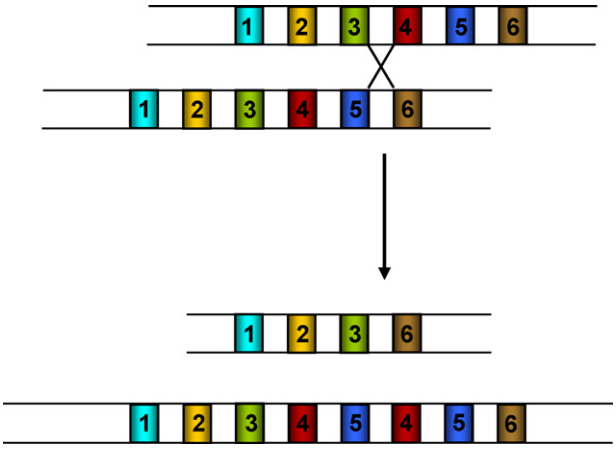

Fig. 3. Model of evolution by unequal crossing over during meiosis. Two sister chromatids are depicted at the top, misaligned with respect to allelic pairing; this erroneous non-syntenic alignment likely occurs between regions displaying a high degree of homology, for example among the repetitive sequences such as LINE1 elements that are abundant within the *Ly49* region. Different genes are numbered accordingly. Shown below are the resolved daughter structures following crossing over and recombination, whereby two novel haplotypes are generated: a deleted or small haplotype having fewer genes (e.g., BALB/c), and an expanded haplotype containing tandem duplication(s) of single or multiple genes (e.g., 129S6, *Ly49q-e* duplications; or NOD, *Ly49i-g-p-pd* duplications). The resulting daughter chromatids would be inherited by offspring, undergoing further evolutionary diversification thereafter.

2.3. 129

The next mouse *Ly49* haplotype to be mapped and sequenced was the 129S6 strain (although all 129 sub-strains tested to date have the same *Ly49* gene set). The 129 haplotype contains 19 *Ly49* genes and its characterization provided the first direct evidence that mouse *Ly49* haplotypes display considerable heterogeneity [16,17]. In fact, the 129 cluster contains multiple *Ly49* genes that have no syntenic allelic counterpart in the B6 cluster (Fig. 1). A genetic snapshot of evolution in motion may be found within the 129 haplotype, where recent group duplications are evident but have not yet been subject to significant genetic divergence or opportunistic deletion of unnecessary genes. Interestingly, the *Ly49q-e* framework gene pair appears to have been duplicated twice in the 129 cluster, leading to the birth of novel $Ly49q_2$-ec_2 and $Ly49q_3$-ec_1 gene pairs. The most recent duplication likely involves the $Ly49ec_1$ and $Ly49ec_2$ genes, which are almost identical. The $Ly49q_2$ and $Ly49q_3$ genes are also very similar and even share a disabling deletion of exons 6 and 7. The theoretical duplication of whole multigenic regions can be explained by the process of unequal crossing over during meiotic recombination (Fig. 3). As with the B6 *Ly49* cluster, most of the functional 129 *Ly49* genes code for inhibitory receptors ($Ly49q_1$, *e, v,* ec_2, *s, t,* i_1, *g, o*), with only three functional stimulatory paralogs (*Ly49r, u, p*). Also similar to the B6 cluster, the 129 haplotype contains many pseudogenes [17]. The large number of pseudogenes possessing intact promoter regions and a full set of potential exons is intriguing. One possible advantage for their retention is their ability to serve as templates in the generation of new genes by the process of gene conversion (GC): this phenomenon is documented to occur during B cell development, whereby immunoglobulin variable gene segments may be diversified somatically; GC has also been proposed to occur within the human HLA region, where some 96/224 genes are not predicted to be expressed or translated [27]. In any case, despite an expanded *Ly49* repertoire and the existence of multiple stimulatory Ly49 isoforms, 129-strain mice remain susceptible to MCMV infection, as only the $Ly49I_1{}^{129}$ inhibitory receptor has been shown to interact with the m157 immunoevasin in this strain [25]. Presumably, this is the intended target of the m157 "decoy" gene product, as B6 mice are rendered susceptible to infection by *m157*-mutant MCMV isolates [28]. Notably, the 129 *Ly49* haplotype appears to be shared among the 129P3, 129X1, 129S1, 129S6, C57L/J, C57BR/cdJ, SJL/J, and FVB/N mouse strains.

2.4. BALB

The BALB/c mouse possesses the smallest known mouse *Ly49* haplotype, containing only eight genes (Fig. 1). This includes only a single candidate pseudogene, *Ly49y*, which possesses a complete coding region, but has not yet been detected at the transcript level (at least not in NK cells). This minimal haplotype consists of all six functional framework inhibitory receptor genes (*Ly49q, e, i, g, c, a*) and only a single functional stimulatory receptor gene (*Ly49l*) [29]. Unlike all other known mouse *Ly49* haplotypes, the BALB/c genome contains no *Ly49h*-related genes, and it was thus instrumental in identifying *Ly49h* as the locus responsible for imparting resistance of B6 mice to MCMV infection (also designated the $Cmv1^r$ locus) [30-33]. Of note, the BALB haplotype is shared among the DBA, C3H/He, CBA/J, and A/J mouse strains.

2.5. NOD

The NOD/ShiLtJ *Ly49* cluster is the largest *Ly49* gene set sequenced to date, with 21 discernable genes (Fig. 1) [34]. This newly sequenced *Ly49* cluster retains interesting regions highly similar to those of the 129 and B6 clusters. It is primarily characterized by a large duplication of the $Ly49i_1$ to *Ly49p* region found in the 129S6 cluster, which in turn is separated by duplications of $Ly49i_1$ and *Ly49p*. Thus, the presence in the NOD *Ly49* haplotype of three *Ly49i* and *Ly49p* genes and two *Ly49g* and *Ly49pd* genes can be explained by tandem duplications followed by a localized deletion. This dynamic cassette bisects the centromeric and telomeric *Ly49* regions, which are otherwise quite similar to the B6 cluster. Interestingly, the NOD *Ly49* cluster encodes the largest number of stimulatory Ly49 receptors identified to date. However, this conceptually increased activation capacity does not correlate with elevated NK function in NOD mice in comparison to NK cells from other mouse strains. In fact, NOD NK cells display diminished killing of CHO cells compared to B6 NK cells [34,35], even though they retain the *Ly49d* locus that defines the capacity to directly lyse CHO cells (also designated the *Chok* locus [36–38]). Furthermore, despite their retention of *Ly49p* and *Ly49h* alleles, NOD mice are susceptible to MCMV infection. These findings may be explained by polymorphisms in $Ly49U/H^{NOD}$ that may abrogate m157 recognition, reciprocal polymorphisms in the $Ly49I^{NOD}$ isoforms that may augment m157-mediated "decoy" inhibition, or additional polymorphisms in the $Ly49P^{NOD}$ isoforms and/or the $H\text{-}2D^{NOD}$ allele that may disrupt the epistatic synergy between these latter gene products [24,25,28,39–44]. Alternatively, uncharacterized cytotoxicity or signaling pathway defects in NOD NK cells may account for their hypofunctional responses to CHO cells and MCMV infection.

2.6. Rat Ly49

A single rat *Ly49* haplotype has been deduced from the complete genome sequencing of Brown Norway (BN) rat strain. In comparison to the mouse *Ly49* clusters, the rat *Ly49* haplotype is considerably larger and more complex, with estimates of gene numbers between 33 and 36 independent loci (nominally 34) [45–47]. Interestingly, a few novel rat *Ly49* gene products exemplify a third functional isoform: these receptors possess a dual, chimeric stimulatory-inhibitory structure, containing both a positively charged trans-

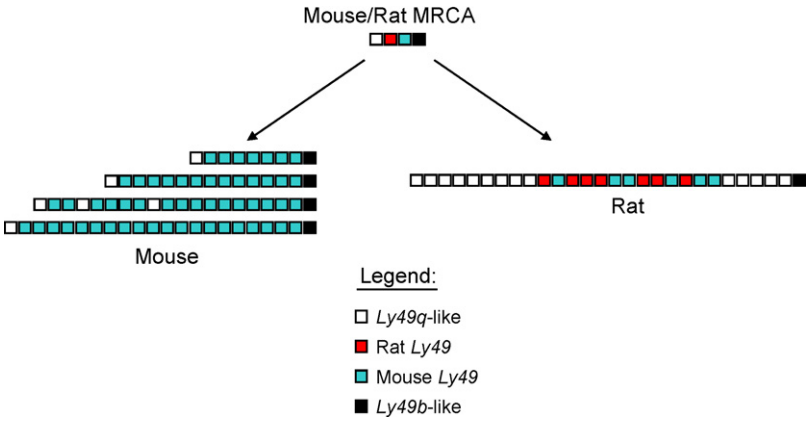

Fig. 4. Rodent *Ly49* haplotype evolution. Known mouse and rat *Ly49* haplotypes evolved from a most recent common ancestor (MRCA) are displayed as a series of squares. Each square represents a separate *Ly49* gene in representative gene orders from selected strains; however, the diagram is not to scale. All known rodent *Ly49* can be grouped into one of four categories as shown in the legend. Fragmentary *Ly49* pseudogenes were not included.

membrane arginine residue, presumably for DAP12 adaptor association, as well as a consensus cytoplasmic ITIM [46]. Phylogenetic analyses of the rat and mouse Ly49 coding regions have revealed the existence of some "mouse-like" *Ly49* in the rat genome [45–47]. Accordingly, rodent *Ly49* can be divided into four basic groups: (i) *Ly49b*-like; (ii) *Ly49q*-like; (iii) mouse *Ly49*; and (iv) rat *Ly49* (Fig. 4). The rat genome contains all four of these subclassifications, and it appears that the rat *Ly49q*-like genes have expanded by duplication several times. It is unknown whether any of these *Ly49q*-like genes are expressed in myeloid lineage or plasmacytoid dendritic cells, akin to mouse *Ly49q* [48–50]. Nonetheless, it is likely that the rat *Ly49* are also highly variable in gene sequence and gene numbers among different inbred rat strains [46,51]. Further genomic analysis of other rat strains is necessary to determine their gene content, organization, and whether or not framework infrastructures exist.

2.7. Human Ly49

In a classical case of convergent evolution, the *KIR* genes emerged in humans as the dominant MHC-I-specific receptors on NK cells. However, the human genome does contain a single *Ly49*-like (*LY49L*) pseudogene [52], demonstrating that a common ancestor presumably shared both the *Ly49* and *KIR* systems. Further evidence in support of this comes from the orangutans, which utilize both receptor systems [53,54].

3. Repetitive elements: drivers of evolution or just 'fill'?

The *Ly49* regions of the B6 [19], 129 [17], BALB [29], and NOD [34] strains are densely populated with repetitive sequences, especially LINE1 elements and to a lesser extent LTR and SINE repeats. In fact, repetitive elements constitute almost 50% of the total nucleotide composition of each of the four *Ly49* gene clusters. This incidence is greater than their combined prevalence across the whole mouse genome, in which they constitute approximately 39% of all sequences [55]. In all characterized mouse *Ly49* regions to date, LINE1 elements in particular make up 36% of the total sequence composition, which is significantly higher than their 19% composition over the whole mouse genome [55]. Although interspersed across the entire *Ly49* region, LINE1 elements are especially concentrated in the *Ly49* intergenic regions, or inserted within introns (between exons 5-6 and 6-7, or sometimes [−1a]-1). In addition, the large intergenic region separating *Ly49q-e* from the rest of the *Ly49* cluster contains an unusually high number of LTR elements in all haplotypes. This region has been hypothesized to act as a regulatory insulator barrier for *Ly49q* and *e*, which have distinct hematopoietic expression patterns compared to other *Ly49* [48,56,57].

Mobile elements may facilitate genome evolution in a number of ways that can be either destructive or advantageous [58]. For example, LINE1 insertions may be responsible for nucleating unequal homologous recombination events that lead to tandem gene duplications. This appears to be true for the *Ly49h*-like subfamily in B6 mice, which contains a tandem *Ly49k-h-n* cluster (Fig. 1). Similarly, in the 129S6 genome, the *Ly49pd-p* segment possesses areas of high internal sequence homology, and the gene pair is most likely the result of tandem duplication. Unequal crossing over may also lead to duplications of larger gene groups, as seen for the *Ly49q-e* triplication in 129 mice and the *Ly49i-g-pd-p* duplication in NOD mice (Fig. 1).

In addition to intact gene duplication, it is also possible that unequal homologous recombination at repetitive sequences, such as LINE1 elements, may permit the evolution of 'hybrid' *Ly49* by facilitating internal recombination between exon groups that code for functional domains (such as between exons 2-3 and 4-5-6-7). This phenomenon was first noted for *Ly49p*, which appears to represent the product of *Ly49a* and *Ly49d* [59]. Similarly, *Ly49l* can be described as a hybrid of *Ly49g* and *Ly49d*, and *Ly49h* can be described as a hybrid of *Ly49d* and *Ly49i* (Fig. 5)

In comparison, the *Nkrp1* region of B6 mice has a similar size and gene density to the *Ly49* cluster [8,9], and appears to possess an equal percentage of repetitive elements at approximately 52%. However, a major difference here lies in the proportion of LINE1 elements, which constitute only ~21% for the entire *Nkrp1–Clr* interval (S.B. and A.P.M., unpublished observations). This is comparable to the genomic average of 19%, and significantly less than the *Ly49* cluster average of 36%. While the make-up of repetitive elements is similar for the *Ly49* regions of all known strains, independent of the size of the cluster, this information is not available for the *Nkrp1–Clr* haplotypes of non-B6 strains, which have yet to be fully sequenced [8,9]. Based on a similar predicted cluster size, due to the conservation of genes between the BALB/c and 129 *Nkrp1–Clr* regions (J.R.C. and A.P.M., unpublished observations), their prevalence of repetitive elements is likely to be very similar. It is tempting to speculate that the overall variability and relatively rapid evolution of the *Ly49* region, compared to the *Nkrp1* region as a whole [8,9], may be linked to the intrinsic density of LINE-1 elements. These repetitive sequences may act as 'sliding' regions of homol-

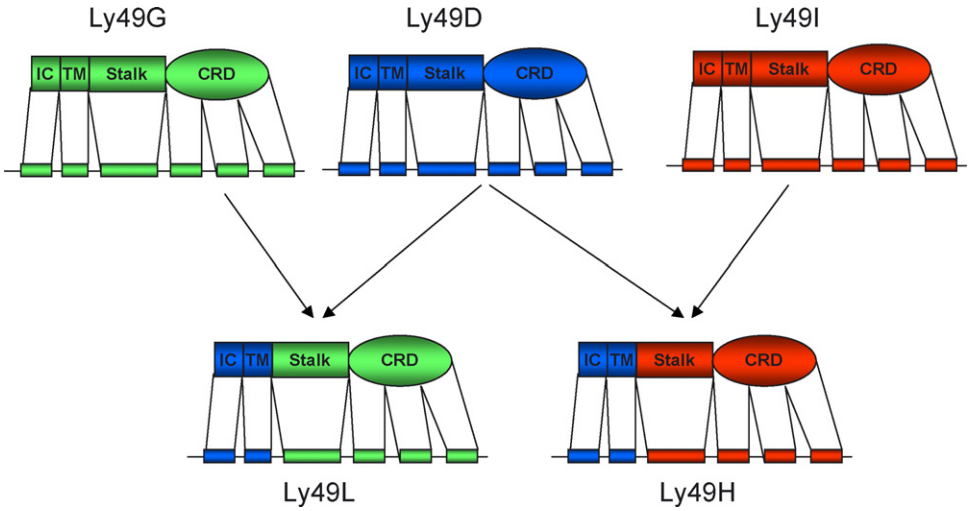

Fig. 5. Model for the evolution of 'hybrid' Ly49 receptors. The coding and non-coding regions (exons and introns) of different *Ly49* genes are shown with their corresponding protein domains above each gene. Depicted at the top are different canonical *Ly49*, which can be recombined in pairs to form novel "hybrid" *Ly49* shown below. The exons coding for protein domains may be recombined to form new chimeric *Ly49* genes, each retaining the cognate specificity of one parental gene and the signal transduction capacity of the second parental gene. For example, a *Ly49l* ancestor can be generated from a recombination event involving the upstream region of *Ly49d* and the downstream region of *Ly49g*, while a *Ly49h* ancestor can be generated from the upstream region of *Ly49d* and the downstream region of *Ly49i*, respectively. The novel hybrid *Ly49* diverges independently following the recombination event. Note, such events can be explained by unequal crossing over and/or gene conversion. IC, intracellular; TM, transmembrane; CRD, carbohydrate recognition domain.

ogy that promote unequal crossing over and subsequent creation of new *Ly49* haplotypes (Fig. 3).

4. The Nkrp1 system: polymorphic receptors for linked monomorphic Clr ligands?

It is apparent that variable gene content and organization are not always features of NKR gene families. Indeed, the Cd94–Nkg2 family was previously shown to be well conserved [60], and recent evidence indicates this holds true for aspects of the Nkrp1 recognition system [8,9].

4.1. Mouse Nkrp1–Clr

The mouse *Nkrp1–Clr* interval lies centromeric to the *Ly49* gene cluster, at the opposite end of the NKC on mouse chromosome 6 (Fig. 6) [4,9,14,61–63]. Here, members of the *Nkrp1* family are found interspersed among the distinct yet related *Clr* (*Ocil/Clec2d*) gene family [64–67]. What sets this system apart from the Ly49 system is that members of the Clr family serve as ligands for members of the Nkrp1 family [68,69]; thus, the receptor–ligand cluster is genetically linked and normally co-inherited *en bloc* [4,9]. Conceptually, this is expected to have a profound effect on the co-evolution of functionally interacting specificities. Indeed, recent work in our labs has demonstrated that the B6, BALB, and 129 *Nkrp1–Clr* haplotypes are much more conserved than their respective *Ly49* counterparts (Fig. 7) ([8]; J.R.C. and A.P.M., unpublished observations). In fact, their copy numbers, orientation, and gene order are completely conserved between the B6, BALB, and 129 strains, resulting in allelic parity at each locus. Superficially, this outcome may have been predicted, due to the tight genetic linkage of the receptor and ligand genes: the *Nkrp1b* and *Clr-b* gene products functionally interact, as do the products of the *Nkrp1f* and *Clr-g* loci [68,69]. These cognate specificities might be expected to negatively impact the evolutionary rate of accumulated polymorphisms, as mutations in either the *Nkrp1* or *Clr* genes might disrupt functional receptor–ligand interactions. Nonetheless, there remain conspicuous exceptions to this inherent fidelity.

Notably, two of the most tightly linked and closely related *Nkrp1* loci form a hotspot of diversity within the center of the *Nkrp1–Clr*

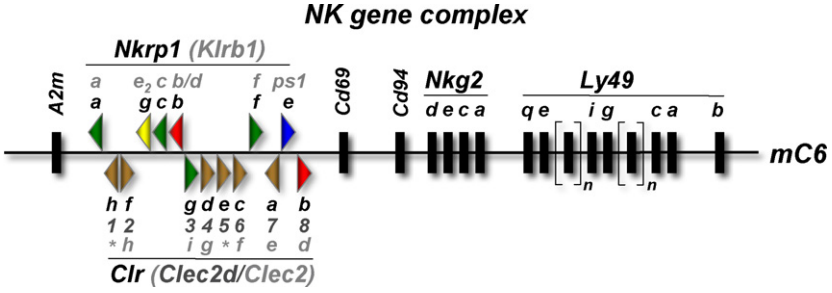

Fig. 6. Model of the mouse NKC highlighting the *Nkrp1–Clr* interval. The NKC on mouse chromosome 6 is depicted in a centromeric to telomeric gene order, with selected gene annotations shown. The *Nkrp1* (top) and *Clr* (bottom) genes are depicted as triangles, to indicate gene orientation, and in color, according to the function of their gene products, as follows: (i) stimulatory NKR-P1 receptors (and their cognate Clr ligands) are shown in green; (ii) inhibitory NKR-P1 receptors (and their cognate Clr ligands) are shown in red; (iii) NKR-P1 receptors of current undocumented expression or function are shown in yellow; (iv) possible non-coding *Nkrp1* pseudogenes are shown in blue; and (v) Clr ligands of current undocumented expression or function (or possible non-coding *Clr* pseudogenes) are shown in beige. All other genes are depicted as black boxes. Alternative nomenclature. The gene order of *Nkrp1 (a, g, c, b, f, e)* is equivalent to *Klrb1 (a, e_2 [or e-like], c, b/d, f, ps1)* in the mouse genome; similarly the gene order of *Clr (h, f, g, d, e, c, a, b)* is equivalent to *Clec2d (1, 2, 3, 4, 5, 6, 7, 8)*, or *Clec2 (*, h, i, g, *, f, e, d)* in the mouse genome, where (*) represents an undefined gene name. Note: the *Nkrp1b* and *Clr-b* gene products interact, as do those of *Nkrp1f* and *Clr-g*. Figure is not to scale.

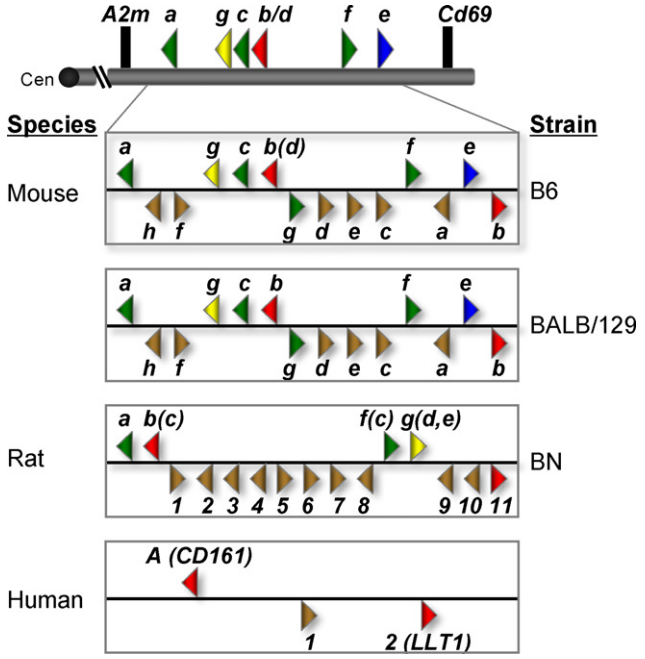

Fig. 7. Known *Nkrp1–Clr* haplotypes in different mouse strains and species. The genomic organizations of known mouse *Nkrp1–Clr* haplotypes (C57BL/6, BALB/c, 129S6), the single known rat *Nkrp1–Clec2d* haplotype (BN/SsNHsdMCW strain), and the known human *NKRP1–CLEC2D* haplotype are shown. Alternative nomenclature. The mouse gene order of *Nkrp1 (a, g, c, b, f, e)* and *Clr (h, f, g, d, e, c, a, b)* are shown, with the exception that *mNkrp1b*^{B6} is also known as *mNkrp1d* in the B6 strain. The rat gene order of *Nkrp1 (a, b, f, g)* and *Clec2d (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11)* are shown, with the following exceptions: *rNkrp1f* and *rNkrp1g*, putative orthologs of *mNkrp1f* and *mNkrp1g*, are also known as *rKlrb1c* and *rKlrb1d*, respectively; *rNkrp1b*^{PVG} and *rNkrp1g*^{PVG} are also known as *rNkrp1c* and *rNkrp1e*, respectively, in the PVG strain. The human gene order of *NKRP1A* and *CLEC2D (1, 2)* is shown, with the alternatives that *hNKRP1A* is also known as *KLRB1A/CD161*, and *hCLEC2D2* is also known as *LLT1*; *hCLEC2D1* is a pseudogene. Note: the products of the following genes are known to interact: *mNkrp1b* (*mNkrp1d*) with *mClr-b*; *mNkrp1f* with *mClr-g*; *rNkrp1b* with *rClec2d11* (*rClr-b*); *hNKRP1A* with *hCLEC2D2* (*LLT1*). Figure is not to scale.

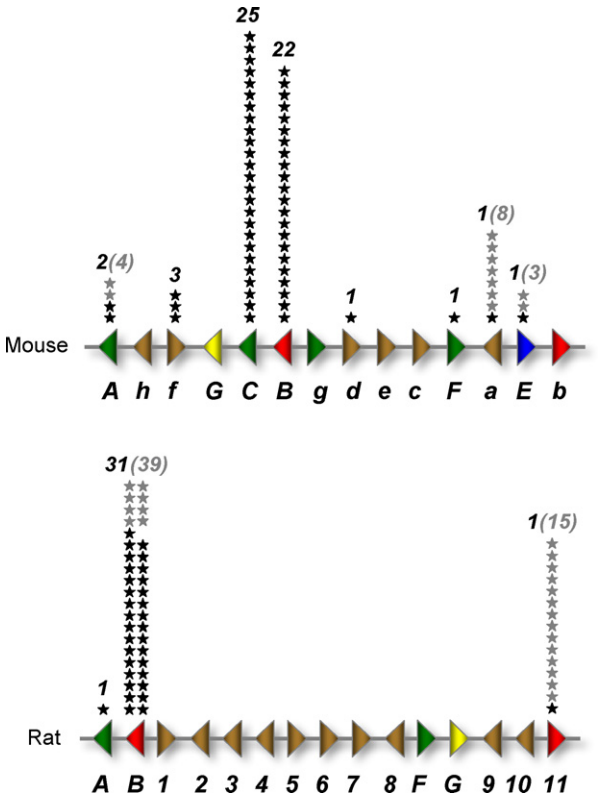

Fig. 8. Coding sequence polymorphism index of the known mouse and rat *Nkrp1–Clr* genes. Identified genetic polymorphisms leading to coding sequence alterations in the known mouse and rat *Nkrp1* and *Clr* gene products were enumerated and plotted as stars (⋆) above the corresponding genes. Mouse and rat *Nkrp1* are denoted in capital letters, while mouse *Clr* are denoted in lowercase letters, and rat *Clec2d* (rat *Clr*) are denoted numerically. Each star (⋆) represents a position in the corresponding gene that exhibits at least one nucleotide polymorphism leading to alteration of the putative protein coding sequence. Note: (i) grey stars in the mouse diagram represent coding sequence polymorphisms observed in strains other than the three primary characterized strains (B6, BALB, 129); (ii) grey stars in the rat diagram represent coding sequence polymorphisms observed in a single rat strain (TO), in which both the *rNkrp1b* and *rClec2d11* (*rClr-b*) genes appear to have diverged significantly.

cluster (Fig. 8). In particular, the *Nkrp1c* and *Nkrp1b* stimulatory and inhibitory paralogs are strikingly divergent between the B6, BALB, and 129 strains, exhibiting a total of 25 or 22 allelic coding substitutions, respectively (Fig. 8) [8]. This intrinsic sequence variability has had a significant impact on our understanding of the system. Indeed, *Nkrp1c* polymorphisms were responsible for the fortuitous discovery of this prototypical NK receptor, based upon its ability to elicit a strong alloantibody response upon challenge of BALB/c-related (C3H) mice with B6-related (CE) splenic NK cells [70,71]. In addition, the *Nkrp1b* locus is sufficiently divergent in the B6 genome that it has been mistakenly given a distinct gene annotation (i.e., *Nkrp1d*), rather than a conventional allelic designation (e.g., *Nkrp1b*^{B6}) (Fig. 7) [72–74]. Collectively, the *Nkrp1c-b* polymorphisms provide an immunological basis behind the definition of the serological NK1.1 marker [75], and explain discrepancies in redirected lysis by NK cells from different mouse strains: specifically, *Nkrp1c*^{B6} encodes a stimulatory NK1.1^{+} receptor [61,76], *Nkrp1b*$^{Sw/SJL/FVB/CD-1}$ encode inhibitory NK1.1^{+} receptors [8,72,73,77], and the *Nkrp1c*$^{BALB/129}$ and *Nkrp1b*$^{BALB/129}$ gene products are NK1.1^{-} by definition [9,72,76].

Notwithstanding the localized divergence of these two adjacent *Nkrp1* genes, the remarkable conservation of the surrounding *Nkrp1* and *Clr* genes provides stark contrast, as several loci in the interval are nearly indistinguishable between the three mouse strains (Fig. 8). Moreover, despite their polymorphic nature, the gene products of the *Nkrp1b*^{BALB} and *Nkrp1b*^{B6} (*Nkrp1d*) alleles both exhibit the same inhibitory function and specificity for their cognate ligand, Clr-b, which is invariant between these and several other strains [72]. This dichotomy between altered receptor sequence versus conserved ligand sequence and function demonstrates that positive selection pressure likely exists to retain an intact "self-specific" receptor–ligand interaction, even in the face of significant receptor polymorphism [9]. Reciprocally, while the ligand for the *Nkrp1c* gene product remains unknown to date, the *Nkrp1c* locus seems to have co-evolved in step with *Nkrp1b* (Fig. 8); this suggests that the two paralogs may share overlapping ligand specificity, yet counterbalance one another functionally. In any case, they appear to be evolving under the same selection pressure. While the functional and evolutionary ramifications of such a rapid and localized gene diversification remain to be fully elucidated, clues to one possible driving force, and perhaps the causal factor behind the diversity, have recently come from studies in the rat system.

4.2. Rat Nkrp1–Clr

The rat *Nkrp1–Clr* system is arguably simpler, and yet deceptively more complex, compared to the mouse system (Fig. 7). Notably, the number of receptor genes is reduced from six *mNkrp1* to four *rNkrp1*, while the number of ligand genes is increased from eight *mClr* to eleven *rClr* (also designated *rClec2d*) [9,64]. Of these, all four *rNkrp1* loci may be functional, whereas only four of six

mNkrp1 genes are known to encode functional proteins [9,64]. Furthermore, up to nine *rClr* loci may yield intact gene products, compared to possibly only four *mClr* genes [9,64,65,67]. Understanding of the rat system is also complicated by inconsistencies in nomenclature, which arise in part due to the assignment of gene names variably by order of discovery [78–80], order in the genome [64], or attempts to decipher true orthology or at least functional homology based upon the mouse system [9,10]. We will attempt to clarify these issues here.

As the *rNkrp1a* gene is the prototype of the *rNkrp1* family [78], it also lies most centromeric in the genome [64], and it may share both functional homology and true orthology with *mNkrp1a* [9,10], we shall nucleate the family with this gene. From here, the gene order is similar to that based upon receptor discovery, namely *Nkrp1a-b-c-d* (or *Klrb1a-b-c-d* in the BN rat genome) [8,9,64,78,79]. However, there have been a number of historical nomenclature discrepancies in the literature, including the following: (i) a highly polymorphic *rNkrp1b* allele (PVG strain; DQ157010) has been designated *rNkrp1c* [80], similar to the *mNkrp1b*^{B6} versus *mNkrp1d* allelic polymorphism issue [8]; (ii) the *rKlrb1c* gene product (likely *mNkrp1f* ortholog) has alternate Genbank references as *rNkrp1b/d* (NM_001085403 [81]); and (iii) the *rKlrb1d* gene product (likely *mNkrp1g* ortholog) has alternate Genbank references as *rNkrp1e* (NM_001085405; DQ157011-2). Notwithstanding these ambiguities, based upon functional homology and deduced orthology, the rat genome designations of *Klrb1a-b-c-d* can be reconciled with the mouse system to the following order: *Nkrp1a/c-b-f-g* (Fig. 7) [10]. While these tentative assignments of putative orthology require further confirmation from functional studies, the designations prove useful here in that they allow better modeling and prediction of evolutionary significance.

On the other hand, the rat *Clr* (*rClec2d*) gene organization has proven difficult to interpret (Fig. 7). It is readily apparent that a rapid expansion and limited contraction led to several genomic *rClec2d* duplications [64]; yet which genes constitute orthologs is not forthcoming in many cases. Based upon intrinsic homology, the genes assigned *rClec2d1-2-3* appear to mirror the *rClec2d6-5-4* genes, respectively; however, their collective genomic organization and orientation is not straightforward, and they are all too similar to one another to assign putative orthology with mouse counterparts (although some are most likely *Clr-g*-like) [10,64]. In contrast, *rClec2d11* is quite clearly orthologous to *mClr-b*, based on genomic position, orientation, sequence homology, and functional interaction with the *Nkrp1b* gene product [10,64]. Likewise, working inwards, *rClec2d10* is most likely an *mClr-a* ortholog, while the *rNkrp1g–rClec2d9* segment appears to bear close resemblance to an inverted and displaced *mNkrp1g–mClr-f* counterpart [10,64]; in turn, the conserved tight linkage of this latter gene pair in both the mouse and rat genomes may be suggestive of a cognate functional interaction. Finally, the remaining two loci (*rClec2d7-8*) appear to represent pseudogene fragments (at least in the BN rat strain) [10,64].

Given these tentative assignments, a few salient observations become apparent. First, the *rNkrp1a–b* and *rNkrp1f–g* segments appear to represent two distinct pairs of paralogous receptor genes arranged in a head-to-tail fashion (Fig. 7). Second, each cassetted pair appears to encode both stimulatory and inhibitory receptor isoforms, which as a consequence may exhibit overlapping ligand specificities [10]. Third, the collinear orientation of each gene pair could enable rapid diversification by gene conversion or unequal crossing over, which in turn could potentiate a prompt evolutionary switch in function. This genomic organization of opposing (yet balanced) receptor gene pairs may be ideally suited to defense against viral immunoevasins: (i) an advantageous inhibitory viral "decoy" ligand could be rapidly counterbalanced and targeted for direct stimulatory recognition; (ii) viral interference with stimulatory ligand upregulation could also inadvertently interfere with inhibitory ligand expression. On the other hand, genetic linkage of the *Nkrp1* and *Clr* genes permits stable co-evolution of functional receptor–ligand pairs to allow for host maintenance of cognate specificities. Collectively, these features may impact the evolution of resistance and susceptibility to pathogens.

4.3. Nkrp1 polymorphism revisited

It is tempting to speculate that *rNkrp1a* may actually be orthologous to *mNkrp1c* (or to both *mNkrp1a/c*). This is supported by the tight linkage and collinear arrangement of the *rNkrp1a-b* and *mNkrp1c-b* gene pairs (Fig. 7). Furthermore, recent evidence indicates that like the *mNkrp1* diversity hotspot, the *rNkrp1* interval appears to be subject to a high degree of focused polymorphism (Fig. 8) [8–10]. In particular, both the mouse and rat *Nkrp1b* inhibitory receptor genes display such remarkable divergence that alleles in both species have been mistakenly given novel gene designations (i.e., mouse *Nkrp1b*^{B6} = *"Nkrp1d"* [74], and rat *Nkrp1b*^{PVG} = *"Nkrp1c"* [80]). Moreover, in both cases the corresponding ligand genes, *mClr-b* and *rClec2d11*, remain invariant between strains (exceptions noted in Fig. 8) [8–10]. In fact, most of the surrounding *Nkrp1–Clr* genes remain conserved between strains in both species (Fig. 8). Why should a genetically linked cognate receptor–ligand pair selectively accumulate polymorphisms in the receptor genes but not the corresponding ligand gene? One likely possibility is that the receptor is under selection pressure to change, while the ligand remains neutral to maintain "self"-integrity. An obvious scenario in which this could occur is under pathogen-driven selection, whereby a virus may evolve a "decoy" for the inhibitory receptor; in turn, the host counter-evolves loss-of-function inhibitory receptor polymorphisms (which lose decoy specificity but maintain "self"-recognition). Of course, the host could also counter-evolve gain-of-function stimulatory receptor polymorphisms (with decoy specificity, but not host crossreactivity). Yet despite the similarities between the mouse and rat systems, of the two *Nkrp1b*-linked stimulatory paralogs, only *mNkrp1c* (but not *rNkrp1a*) shares the directed polymorphism. This may suggest that the mouse system at one time underwent successful pathogen-driven evolutionary change. As discussed below, recent evidence from the rat *Nkrp1* system confirms this scenario.

4.4. Evolution and counter-evolution: CMV immunoevasins and the rat Nkrp1 system

During the search for mouse *Clr-b* homologs, an interesting cDNA sequence emerged from studies using a defined isolate of rat cytomegalovirus (RCMV-English) [82]. This viral gene product, termed RCTL (RCMV C-type lectin-like), exhibits a number of striking features: (i) RCTL shares a high degree of sequence homology with both mouse Clr-b (~60% nucleotide and ~50% coding identity) and the rat Clr-b ortholog, rCLEC2D11 (~66% nucleotide and ~60% coding identity); (ii) *rctl* is a spliced gene, containing five exons and four introns, the same genomic structure as the mouse and rat *Clr-b* genes; and (iii) the RCTL ectodomain is highly conserved with rClr-b, but the cytoplasmic region is divergent, suggesting similar specificity yet dissimilar function [9,10,82]. Collectively, these features suggested that RCTL may have evolved as an RCMV "decoy" ligand for the rat NKR-P1B inhibitory receptor [9].

Indeed, this turned out to be the case, but a number of additional facets to the story were further uncovered [10]. First, host Clr-b expression appears to be rapidly lost at both the transcript and protein levels upon RCMV infection, in a manner suggestive of a defensive innate host response. Second, RCTL rapidly replaces

host Clr-b at the cell surface, demonstrating the immunoevasin possesses independent regulation at the protein level. Third, RCTL functions as a *bona fide* decoy ligand, binding directly to the host NKR-P1B inhibitory receptor, but only in selected rat strains (e.g., WAG, PVG, BS, but not SD, BN, F344). Fourth, RCTL directly inhibits NK cytotoxicity *in vitro* and augments RCMV virulence *in vivo*, but again only in rat strains in which the RCTL:NKR-P1B interaction is robust. Finally, RCTL also binds to the host NKR-P1A stimulatory receptor, but only weakly, at least among the strains tested [10].

Taken together, these findings support a dynamic model of host–pathogen interactions, involving evolution and counter-evolution strategies on both sides. RCMV likely acquired the ancestral *rctl* gene (introns intact) from a host cell during a replication cycle [10,82]. In turn, the RCTL gene product provided a selective advantage *in vivo*, serving as a decoy immunoevasin for the rat NKR-P1B inhibitory receptor, evolving over time. Under selection pressure from this viral evasion strategy, the host counter-evolved at least three discernable genetic strategies: (i) accumulate NKR-P1B polymorphisms that avert RCTL decoy binding; (ii) conserve the host Clr-b ligand, and in parallel retain NKR-P1B polymorphisms that maintain a cognate "self" interaction; and (iii) acquire NKR-P1A polymorphisms *de novo* that facilitate direct recognition of RCTL, yet crossreact minimally with host Clr-b. The combined impact of these countermeasures would be expected to allow certain host strains to avert the RCTL viral decoy strategy altogether [10]. In turn, this model provides a rationale behind the localized polymorphism observed in the *rNkrp1a-b* and *mNkrp1c-b* genes (Fig. 8): they are under pathogen selection pressure to diverge. Since *rNkrp1a* displays considerably less polymorphism than *mNkrp1c* [8,10], and the rNKR-P1A alleles tested only interact weakly with RCTL [10], the counterbalancing cycle of host evolution in the rat may still be incomplete, compared to that of the mouse. Thus, the pronounced polymorphism observed at the mouse *Nkrp1c-b* loci may represent an evolutionary remnant of a successful host adaptation to circumvent a past pathogen challenge. The absence of identified lectin-like genes in known laboratory and wild MCMV isolates is consistent with this possibility. Alternatively, the spliced nature of the *rctl* gene [82] suggests analogous MCMV genes may have eluded earlier discovery. In any case, it is quite possible that the ligand for the mouse NKR-P1C receptor may have been, at one time, an MCMV immunoevasin.

4.5. Human Nkrp1–Clr

Unlike the Ly49 system, the Nkrp1–Clr system is functionally conserved in humans (Fig. 7). While confusion shrouded the function of the human *NKRP1A* (*CD161/KLRB1A*) gene product for many years [83], insight from the cloning of ligands for the mouse *Nkrp1* family [68,69] facilitated rapid identification of a human *Clr* ortholog [84,85]. Previously known to human NK cell biologists as *hLLT1* (*hCLEC2D2*) [64,86,87], this gene shares many similarities with *mClr-b* and *rClec2d11*. Importantly, *hLLT1* and *hNKRP1A* are genetically linked and share the same copy number, gene organization, orientation, and greatest sequence homology with the rodent *Clr-b* and *Nkrp1b* genes, respectively (Fig. 7). Moreover, LLT1 is the only known ligand for the solitary hNKR-P1A receptor and its expression on target cells inhibits NK function [84,85], contrary to the majority of published reports suggesting a stimulatory role for hNKR-P1A. On the other hand, LLT1 appears to possess a distinct expression pattern *in vivo* [88], compared to that of rodent Clr-b [10,68]. The discovery of a second putative inhibitory receptor gene (*Nkrp1g*) in both the mouse and rat genomes (Fig. 7) [8–10] could call into question the orthology of the *hNKRP1A–hLLT1* gene pair with the *mNkrp1b–mClr-b* and *rNkrp1b–rClec2d11* loci. However, the rodent *Nkrp1g* gene possesses lower homology to *hNKRP1A* (versus *Nkrp1b*), *Nkrp1g* expression and function remain undocumented, and ligands for an intact *Nkrp1g* gene product remain unknown to date (although two intriguing possibilities are suggested by the conserved tight linkage of the *Nkrp1g–Clr-f* segment, and the paralogy of *Nkrp1g* with *Nkrp1f*, which encodes a receptor for the *Clr-g* gene product) (Fig. 7) [8,9]. Further investigation into the human system is required to determine its function *in vivo*, its potential as a candidate for therapeutic manipulation, and whether or not HCMV has evolved RCTL-like immunoevasins that may function to subvert NKR-P1A-mediated recognition of infected cells.

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