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Mapping of the BALB/c *Ly49* cluster defines a minimal natural killer cell receptor gene repertoire☆

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Abstract

The BALB/c inbred mouse strain is one of the most commonly used for immunological studies and is an animal model for natural killer (NK) cell function during pathogen infection and tumorigenesis. To understand better NK cell function in this strain, the complete BALB/c *Ly49* haplotype was deduced. The BALB/c haplotype spans approximately 300 kb with a gene order and content of *Ly49q*, *e*, *x*, *i*, *g*, *l*, *c*, and *a*. Functional BALB/c alleles of *Ly49q* and *e* were isolated and found to be conserved. The BALB/c cluster represents a minimal haplotype as it contains many fewer functional genes than the 129 or B6 mouse strains. The small number of BALB/c *Ly49* genes is due mainly to an absent group of genes (relative to B6 and 129) between *Ly49x* and *i*, although other smaller deletions are present. These gene deletions provide a genetic basis for the lack of certain *Ly49*-associated NK cell functions in this mouse strain. Finally, the mapping of a third *Ly49* haplotype reveals that the basic murine *Ly49* repertoire is composed of three framework gene pairs (*Ly49q* and *e*, *Ly49i* and *g*, and *Ly49c* and *a*) that are interspersed with variable numbers of strain-specific *Ly49*.

Keywords: BALB/c; NK cell; Receptor; Ly49; BAC contig; Gene mapping

Natural killer (NK)[1] cell killer Ig-related receptor (KIR) repertoires in humans are highly polymorphic due to variable gene content of individual haplotypes and a great degree of allelic diversity [1]. Such variable expression of NK cell receptors and corresponding MHC ligands in individuals is coincident with susceptibility to diseases in which NK cells have been thought to play either a protective or a deleterious role. For example, the presence of *KIR3DS1* and *HLA-B Bw4-80Ile* in HIV^+ individuals correlates with a significant delay in the progression to AIDS [2]. Similarly, activating *KIR2DS1* and *2DS2* has been associated with psoriatic arthritis, while *KIR2DS2* has also been linked with rheumatoid vasculitis [3,4]. Therefore, it would appear that the presence or absence of individual NK cell receptors in individuals might be good prognostic indicators for various disease conditions. As the KIR family in humans is large, it is likely that other disease manifestations will be connected with specific KIR proteins. With respect to clinical translation, the knowledge generated from research in the past decade on human NK cell receptors has allowed researchers to generate successful strategies for the treatment of acute myeloid leukemia [5].

Comparable understanding of murine NK cell receptors will facilitate the application of scientific knowledge to disease conditions as treatment efficacy can be more safely and rapidly ascertained in mice. Therefore, NK cell receptor repertoires in mice must be well defined. In mice, the Ly49 family of C-type lectin transmembrane proteins performs the same role as KIRs in humans with similar ligands (class I MHC), variegated patterns of expression, signal transduction pathway components, and functional outcomes [6]. The C57BL/6 (B6) mouse is the model of choice for many researchers and the total *Ly49*

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[1] *Abbreviations used*: B6, C57BL/6; NK, natural killer; MCMV, mouse cytomegalovirus; KIR, killer cell Ig-like receptor; BAC, bacterial artificial chromosome; PFGE, pulsed-field gel electrophoresis; NKC, NK gene complex; MHC, major histocompatibility complex; HSV, herpes simplex virus.

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gene repertoire has been sequenced in this strain along with the rest of the mouse genome. In a main cluster of approximately 600 kb in the NK cell gene complex (NKC) on mouse chromosome 6, 15 uninterrupted *Ly49* genes are located in tandem, with identical orientation and a centromere-to-telomere order of *Ly49q*, *e*, *x*, *f*, *d*, *k*, *h*, *n*, *i*, *g*, *l*, *j*, *m*, *c*, and *a* [7]. This is followed by *Ly49*-related pseudogenes α, β, and γ and a more distal and differentiated functional gene, *Ly49b*. Only C57BL/6 and C57BL/10 inbred mouse strains are known to possess this *Ly49* haplotype [8].

The only other mouse *Ly49* haplotype defined to date is from the 129 strain [9]. Based on RFLP and flow cytometry with strain-specific anti-Ly49 mAb, the 129 haplotype is shared by SJL, C57L, C57BR, and FVB inbred mouse strains [8,10]. The 129 *Ly49* cluster is composed of at least 18 genes, some of which are known from only a few exons. With some similarity to the B6 haplotype, the 129 gene order is *Ly49q*$_1$, *e*, *v*, *q*$_2$, *e*/*c*$_2$, *l*/*r*, *s*, *t*, *e*/*c*$_1$, *r*, *u*, *u*/*i*, *i*$_1$, *g*, *p*/*d*, *p*, *i*$_2$, and *o* [11]. *Ly49b* has also been identified in this mouse strain. While alleles are identifiable between the two haplotypes, it is evident that the 129 genome contains strain-specific *Ly49* duplications as well as unique *Ly49* genes, and the same is true of the B6 mouse. Furthermore, the MHC-binding patterns of alleles shared by the two haplotypes are similarly divergent [11,12]. This suggests that NK cells in the two haplotypes may be functionally different. Indeed, the two strains of mice have documented differences in their responses to intracellular infection [13], bone marrow transplant rejection [14], and carcinogen-induced tumorigenesis [15]. NK cells have long been implicated as pivotal factors in the outcome of all these conditions.

Similarly, murine cytomegalovirus (MCMV) infection is controlled in part by NK cells. The initial growth of MCMV in the spleen of mice is higher in BALB/c and DBA/2 strains (*Cmv1*^{s}) than in B6 (*Cmv1*^{r}) [16]. Protection or low splenic titer is due to the presence of the *Ly49h* gene in resistant mouse strains [17–19]. Ly49H is able to bind the MHC-like MCMV m157 protein expressed on the surface of infected cells, resulting in NK cell cytotoxicity and cytokine production [20,21]. The absence of the *Ly49h* gene and protein in *Cmv1*^{s} mice allows MCMV titers to attain high levels; this can be prevented in susceptible strains by introduction of a *Ly49h* transgene [22]. In addition to being a model strain for MCMV studies, BALB/c mice are also used as animal models for many immunological studies, including induction of experimental allergic encephalomyelitis and susceptibility to HSV-1 and *Leishmania major* infection [23–25]. The contribution of the BALB/c *Ly49* repertoire to these models is unknown. To understand better the functional potential of NK cells from BALB/c mice, the BALB/c *Ly49* repertoire and gene map were determined.

Results and discussion

A physical map of the BALB/c Ly49 gene cluster

To date, five *Ly49* cDNA sequences are known to be transcribed in BALB/c mice: *Ly49a* (U34890) [26], *b* (AF253057; GenBank Accession only), *c* (U49868) [27], and *g* and *l* (AF307946 and AF307947, respectively) [28]. Compared to the *Ly49* repertoires known for B6 and 129 mice, BALB/c mice have very few known *Ly49*. To determine the total BALB/c *Ly49* repertoire it was decided to construct a gene map for this region of chromosome 6 in BALB/c mice. Bacterial artificial chromosome (BAC) clones from a BALB/c genomic library containing *Ly49* genes were identified by specific hybridization to a probe cocktail representing all of the known *Ly49* subfamilies. The gene content of these *Ly49*$^+$ BAC clones was assessed by PCR with primers specific to exons 3 and 4 of the

Table 1
BALB/c *Ly49* detected by Southern and PCR analysis of BAC clones[a]

	Ly49q 7.6[b]	*Ly49e* 4.0	*Ly49x* 3.0	*Ly49i* 5.4	*Ly49g* 2.7/0.3	*Ly49l* 4.2	*Ly49c* 4.9	*Ly49a* 7.6	(α) 2.4/0.9	(γ) 3.5
321d7										
373f5										
360d21										
347i8										
378f4										
456b23										
410j22										
339b20										
343d13										
340f19										
414f3										
365p9										

[a] *Ly49* genes were detected first in BAC clones by PCR with exon 3- and 4-specific primers followed by sequencing (filled). Sequence-confirmed *Ly49* fragments were then used as probes in Southern analysis of BACs (stippled).
[b] Size in kilobases of BAC clone fragment detected with *Ly49* intron 3-specific probes after *Eco*RI digestion.

known BALB/c *Ly49* (*Ly49a*, *c*, *g*, and *l*). *Ly49b* was not analyzed as it is not closely associated with the other *Ly49* in B6 and 129 mice. In addition, because the *Ly49q* and *e* genes are highly conserved between 129 and B6 mice [9], it was thought likely that these genes would also be present in the BALB/c haplotype and so primers for these genes were also used to PCR-screen the BAC clones. Postamplification, products in the correct size range for intron 3 were cloned and sequenced to confirm gene identity based on exons 3 and 4.

In addition to the known *Ly49a*, *c*, *g*, and *l* genes for this strain, several BAC clones were positive by PCR for *Ly49q* and *e*, and the identity of these genes was confirmed by sequencing. The BACs that were positive by PCR and sequencing for each of the tested genes are indicated in Table 1. No other *Ly49* gene sequences were initially discovered by PCR. The BAC gene content provided by PCR screening allowed the construction of a preliminary relative gene order: *Ly49q*, *e*, *g*, *l*, *c*, and *a*. Overall, the gene order is similar to that of the alleles known for the B6 and 129 haplotypes, although relative to these two strains, there appear to be many genes missing in the BALB/c cluster, especially between *Ly49e* and *g*. To confirm the gene content of individual BACs, BAC DNA was subjected to Southern analysis after digestion with *Eco*RI. Hybridization with each of the exon 3–4 sequence-confirmed PCR fragments for *Ly49q, e, g, l, c,* and *a* firmly established the *Ly49* gene content of each BAC. After each hybridization, the blot was washed at high stringency to reduce cross-hybridization to other closely related genes as much as possible, although closely related genes often cross-hybridized efficiently. The resulting hybridization patterns are shown in Fig. 1A (top). All BAC clones that were positive by PCR analysis presented specific fragment sizes that strongly hybridized to certain probes. The gene content estimated by PCR was confirmed by Southern analysis and is summarized in Table 1.

To visualize all *Ly49* genes, a low-stringency wash was performed after the *Ly49q* hybridization. This allowed the simultaneous detection of many *Ly49* genes due to the almost equal, and relatively low, homology of *Ly49q* to all other *Ly49* genes [9]. In addition to fragment sizes for *Ly49q*, *e*, *g*, *l*, *c*, and *a*, other fragments were also visible

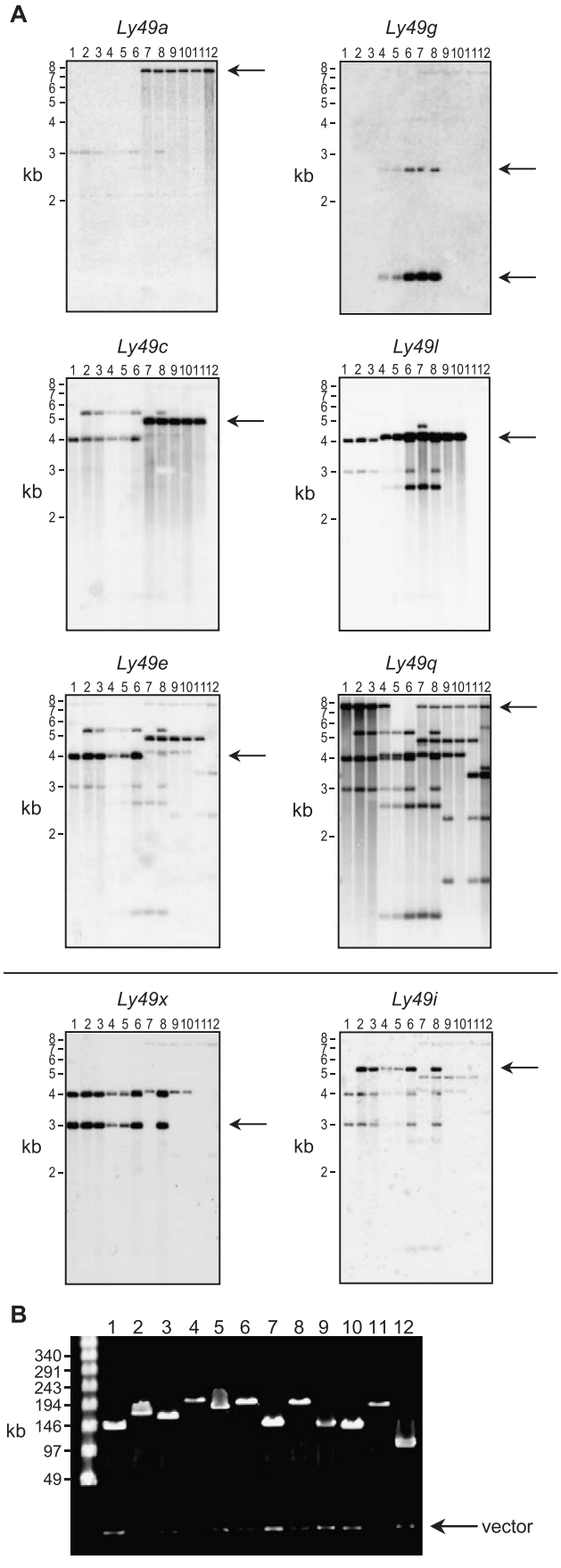

Fig. 1. Characterization of *Ly49*-containing BALB/c BAC clones. (A) BALB/c BACs that were PCR-positive for *Ly49* were digested with *Eco*RI, grouped according to PCR results, and analyzed by Southern hybridization. The type of *Ly49* exon 3–intron 3–exon 4 probe used is shown above each autoradiogram. The same blot, which was stripped between experiments, is shown for all hybridizations. The arrows indicate the fragment containing the *Ly49* exon 3–intron 3–exon 4 fragment hybridizing to the specific probe. (B) BAC DNA was digested with *Not*I and separated on a 1% agarose, 1× TAE gel by PFGE. The excised vector is indicated by an arrow. The lane order is (1) 321d7, (2) 373f5, (3) 360d21, (4) 347i8, (5) 378f4, (6) 456b23, (7) 339b20, (8) 410j22, (9) 340f19, (10) 343d13, (11) 414f3, and (12) 365p9.

at 3.0, 5.4, 2.4, 0.9, and 3.5 kb. The 2.4- and 0.9-kb fragments were always found on *Ly49a*-containing clones and were the same expected *Eco*RI fragment sizes for the α pseudogene from the 129 haplotype, suggesting this was the α pseudogene of BALB/c mice [7] (S.K. Anderson and A.P. Makrigiannis, unpublished observations). As the 3.5-kb fragment mapped even more distally to *Ly49a,* it was provisionally identified as the γ pseudogene (β does not have exons 3 and 4). The 3.0- and 5.4-kb fragments colocalized between *Ly49e* and *g*. Based on the probe that most strongly bound to these fragments (*Ly49a*, 3.0 kb, and *Ly49c*, 5.4 kb), and their location within the cluster, the 3.0- and 5.4-kb fragments were considered to represent potentially the BALB/c alleles of *Ly49v* (or possibly *x*) and *i*, respectively.

Primers were designed to detect the presence of possible *Ly49v* and *i* genes. In addition to amplifying known closely related genes, DNA sequence for exons 3 and 4 of two new BALB/c *Ly49* was detected in BACs predicted to have the 3.0- and 5.4-kb fragments that hybridized strongly to *Ly49a* and *c,* respectively (Table 1). The *Ly49v* primers amplified a new exon 3 sequence containing an arginine residue in the correct location for DAP12 association. The sequence most closely aligned with $Ly49x^{B6}$ pseudogene (also an activator), while exon 4 of this sequence most closely aligned with $Ly49p^{129}$. It was decided to name this new gene provisionally as $Ly49x^{BALB}$. After sequencing, this fragment was used as a probe and hybridized most strongly to the 3.0-kb fragment of *Eco*RI-digested BAC DNA (Fig. 1A, bottom). Interestingly, the *Ly49x* probe also bound quite strongly to a 4.0-kb fragment that was not *Ly49e*, as it was present on the same BACs as *Ly49x*, unlike *Ly49e*. This same *Ly49x*-cross-hybridizing 4.0-kb fragment was also less strongly detected with the *Ly49l* and *q* probes. This suggests that there may be two adjacent *Ly49x*-like genes in BALB/c mice, although attempts to PCR and sequence a separate *Ly49x*-like sequence did not succeed. Furthermore, primers capable of amplifying other *Ly49d*-like (*Ly49d*, *r*, *p*, and *l/r*) genes did not give any products when BALB/c BACs were used as templates.

The *Ly49i* primers amplified an exon 3 and 4 sequence that was almost identical to $Ly49i^{129}$ and was termed $Ly49i^{BALB}$. The $Ly49i^{BALB}$ gene fragment was then used as a probe and was found to hybridize efficiently to the 5.4-kb *Eco*RI fragment previously (and less efficiently) detected with a *Ly49c* probe (Fig. 1A, bottom).

To generate a physical map of the locations of each of the BALB/c *Ly49* genes, the size of each *Ly49*-containing BAC clone was determined by vector backbone excision (*Not*I) and pulsed-field gel electrophoresis (Fig. 1B). The average size of each BAC clone was 174 kb. Integration of BAC clone size and gene content deduced by PCR/Southern analyses allowed the construction of a BAC contig and physical gene map for the BALB/c *Ly49* cluster (Fig. 2). Not counting peripheral pseudogenes, the main *Ly49* gene cluster of BALB/c mice is predicted to span approximately 300 kb, with a minimum gene content and order of *Ly49q*, *e*, *x*, *i*, *g*, *l*, *c*, and *a*. Therefore, even though the individual alleles of the BALB/c haplotype share higher identity with the corresponding alleles of the

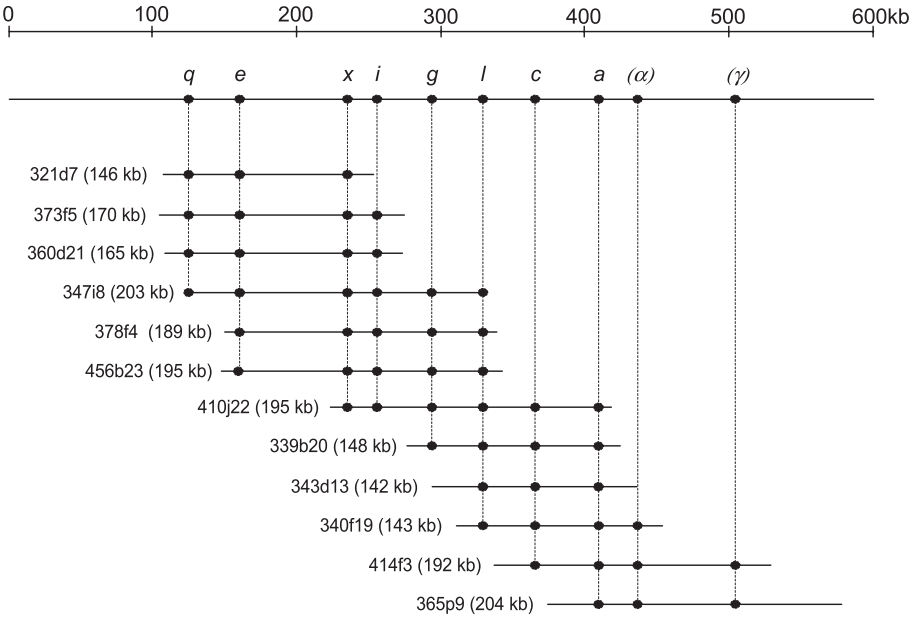

Fig. 2. BAC contig overlap and construction of a physical map for the BALB/c *Ly49* gene cluster. The information from Table 1 and Fig. 1B on BAC *Ly49* gene content and size was integrated to produce a map of the relative locations of all known BALB/c *Ly49* genes. *Ly49b* is excluded from this diagram. BACs are represented by horizontal lines and the name and size of each are given on the left. Each dot represents the start of exon 4 of the indicated gene. The spacing between the BALB/c *Ly49* genes was based on the known distances between the fourth exons of the corresponding B6 alleles. If there was no precedent, spacing was based on the average distance between exons 4 of the *Ly49* genes, which is approximately 36 kb, or physical constraints imposed by the predicted BAC size and gene content.

129 haplotype [29], the overall gene content and order appear to be more B6-like.

To better assess the allelic relationships of the newly identified exon 3 and 4 sequences to B6 *Ly49,* a phylogenetic analysis of all BALB/c and B6 *Ly49* exons 3 and 4 was performed and is shown in Fig. 3. Bootstrap analysis supported the allelic assignments made above, although in some cases confidence levels of some branchings was low due to the high homology within the *Ly49* family and the hybrid nature of some genes [30].

Identification of two new full-length BALB/c Ly49 cDNA sequences

The BALB/c *Ly49* haplotype was found to have exons 3 and 4 of genes that closely resembled *Ly49q*, *e*, *x*, and *i* of the B6 haplotype. To confirm the identity and ascertain the functional status of the new BALB/c *Ly49*, RT-PCR was performed on BALB/c ALAK RNA with primers capable of amplifying the total coding region of the corresponding B6 and 129 alleles. A cDNA sequence for $Ly49e^{BALB}$ was isolated and, relative to $Ly49e^{B6}$, was found to have three single nucleotide polymorphisms over the coding region resulting in one amino acid substitution. The almost complete lack of amino acid sequence variation in *Ly49e* from three different mouse haplotypes suggests an important function for this receptor. A cDNA with seven single nucleotide polymorphisms over the coding region resulting in six amino acid substitutions relative to $Ly49q^{B6}$ was also isolated and termed $Ly49q^{BALB}$. Amino acid substitutions of *Ly49q* and *e* alleles are shown in Table 2. cDNAs for $Ly49i^{BALB}$ and x^{BALB} could not be detected. It is unknown if the $Ly49x^{BALB}$ and i^{BALB} genes are functional or even complete, but the exon fragments identified here did not contain premature stop codons.

Comparison of BALB/c, 129, and B6 Ly49 locus organization

Comparison of the B6 and BALB/c *Ly49* repertoires shows that, unlike the 129 gene cluster, all BALB/c genes had readily identifiable alleles in the B6 haplotype (Fig. 4A). Similar to the 129 and B6 genomic organization, flanking genes were conserved, while the interior genes were much more variable. Also, like the 129 and B6 haplotypes, the BALB/c haplotype had unique structural characteristics. The most impressive was an approximately 200- or 300-kb deletion between *Ly49x* and *i* relative to the B6 and 129 gene maps, respectively. This 200/300 kb insertion contains the *Ly49d*, *h*, and *f* genes (*Ly49r*, *u*, and *s* in 129) in the B6 strain, as well as some 129-specific (*Ly49e/c*$_1$, *e/c*$_2$, *q*$_2$, *l/r*, *u/i*, and *t*) or B6-specific (*Ly49k*)

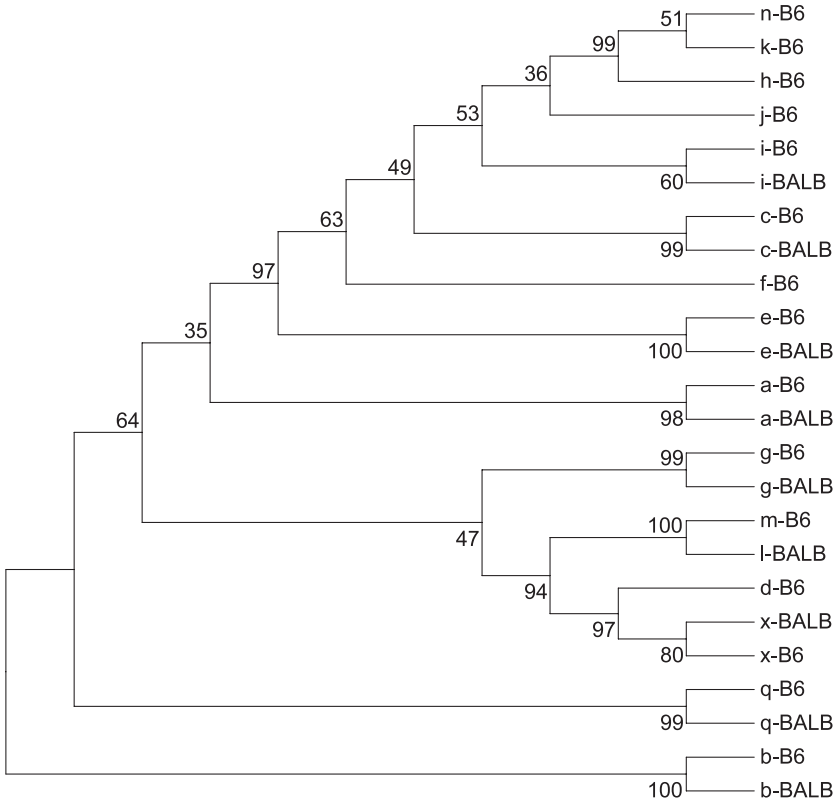

Fig. 3. Phylogenetic analysis of new BALB/c *Ly49*. All exon 3 and 4 cDNA sequences from B6 and BALB/c mice were aligned using ClustalX. Bootstrapping was performed using the PHYLIP software package. B6 cDNA sequences were retrieved from GenBank. Bootstrap confidence values are shown as percentages at each node and are representative of 1000 different bootstrap sets.

Table 2
Comparison of amino acid sequence between alleles of *Ly49q* and *e*

	Domain	Cytoplasmic	Stalk				CRD[c]	
	Amino acid[a]	-[b]	93	102	113	133	229	253
Ly49q	B6	-	N	L	D	V	T	V
	BALB	-	S	P	N	I	K	F
	Amino acid	32		-			-	
Ly49e	129	G		-			-	
	B6	G		-			-	
	BALB	A		-			-	

[a] Position at which a polymorphism is detected.
[b] Amino acid sequence is identical in this domain.
[c] Carbohydrate recognition domain.

genes. Whether this variable region is the result of a deletion in the BALB/c haplotype or an expansion in the B6 and 129 is unknown at this time.

The large deletion within the BALB/c haplotype, relative to the B6 and 129 haplotypes, provides a genetic basis for at least two unique characteristics of BALB/c mice and other strains with the same *Ly49* haplotype. BALB/c NK cells lyse CHO tumor cells much less efficiently than B6 NK cells [31,32]. The recognition of CHO by B6 NK cells is due to the interaction between Ly49D and CHO MHC [33]. Due to the deletion between *Ly49x* and *i,* BALB/c mice have no *Ly49d*-like gene that can function as an activating receptor for CHO tumor cells. Second, also within this "deletion," B6 mice have the *Ly49h* gene, which is responsible for the Cmv1^r phenotype [17–19]. This deletion explains the Cmv1^s phenotype of BALB/c mice [34]. On the other hand, BALB/c mice have a unique and functional activator, Ly49L (likewise with Ly49P from 129 mice), that may prove to be a source of NK cell function and protection that Ly49L (and Ly49P)-deficient strains, like B6, do not possess.

Framework Ly49

Almost all human KIR haplotypes contain at least four common genes, *2DL4*, *3DP1*, *3DL2*, and *3DL3*. *3DL3* and *3DL2* are found on the flanking ends of the cluster, while *3DP1* and *2DL4* are internal. Between these genes, the content of individual haplotypes is highly variable, thus, these four *KIR* genes are referred to as "framework" *KIR* [35]. Likewise, comparison of the BALB/c, 129, and B6 *Ly49* haplotypes has revealed another similarity to KIRs in the form of framework *Ly49*. The *Ly49* framework genes are *Ly49q* and *e*, *Ly49i* and *g,* and *Ly49c* and *a* (Fig. 4B). In the case of *Ly49*, the framework genes come in pairs. In the variable region between *Ly49q* and *e* and *Ly49i* and *g* there can be 1 (BALB/c), 6 (B6), or 10 (129) genes. Similarly, the variable region between *Ly49i* and *g* and *Ly49c* and *a* can contain 1 (BALB/c), 2 (129), or 3 (B6) genes. *Ly49l* was not counted as a framework *Ly49* because only exon 7 remains in the B6 haplotype. Similar to *KIR3DP1,* the *Ly49c* framework gene is a pseudogene in some strains such as 129 ($Ly49i_2^{129}$). Another caveat is that all *Ly49* haplotypes known to date contain at least one functional activator. Before definitive conclusions can be made as to the make-

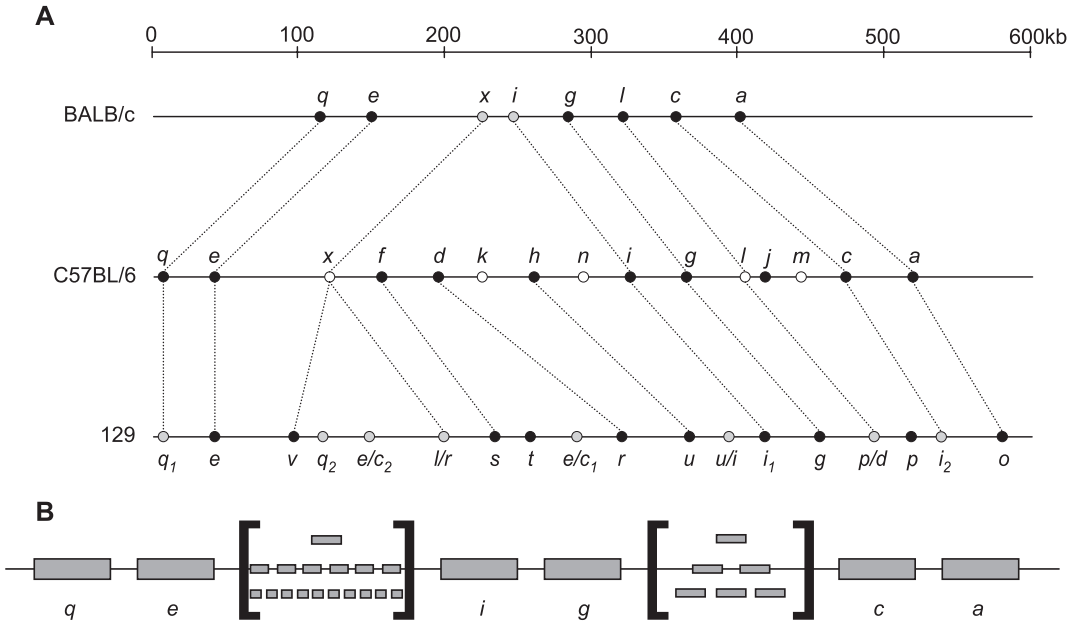

Fig. 4. Comparison of the BALB/c, 129, and B6 *Ly49* gene maps reveals a minimal *Ly49* haplotype. (A) The BALB/c *Ly49* gene map is shown with those known for 129 and B6 strains. All dots represent exon 4, except for *Ly49l* from B6, which is exon 7. The connecting lines between genes of different strains indicate probable allele relationships. The functional status is indicated by the color of the dot: black, functional transcript detected; white, pseudogene; gray, unknown. The pseudogenes beyond *Ly49a* are not shown. (B) The minimal Ly49 haplotype deduced from the common or framework genes from these three mouse strains. Each gray block represents a *Ly49* gene or pseudogene. Note that *Ly49c* and *a* are alleles of $Ly49i_2$ and *o,* respectively, in 129 mice. The two pairs of brackets encompass variable regions within the *Ly49* complex that contain different numbers and types of *Ly49* depending on the haplotype.

up of murine framework *Ly49* genes, more haplotypes will need to be mapped.

Concluding remarks

It would appear that with respect to genomic organization and complexity, the 129 and B6 haplotypes are more closely related to each other than to the BALB/c; however, sequence comparison of conserved genes indicates that the 129 and BALB/c *Ly49* are more closely related to each other than to B6 [9,29]. It is possible that the BALB/c haplotype represents an ancestral *Ly49* organization that underwent a large duplication giving rise to the *Ly49d*- and *h*-containing region, which further evolved, with smaller modifications, into the 129 and B6 haplotypes. A mouse with a *Ly49* genotype without an MCMV-specific activating receptor (Ly49H) is assumed to be at a huge disadvantage in the wild, but it is possible that MCMV may not have been endemic in the region where the BALB/c *Ly49* haplotype existed, or these mice possessed other immune defenses against MCMV that made *Ly49h* unnecessary. One cannot also discount the possibility that in the wild, BALB and B6 *Ly49* haplotypes are maintained in a heterozygous fashion since a single copy of *Ly49h* is sufficient to confer protection to MCMV [18].

Based on a unique assortment of *Ly49* the NOD mouse represents a fourth *Ly49* haplotype. NOD mice are known to express alleles of *Ly49a*, *b*, *d*, *e*, *g*, *m*, *p*, and *w* genes [36]. This haplotype contains genes that are found in 129 (*Ly49p*), in B6 (*Ly49m*), and common to all strains (*Ly49a*, *b*, *e*, and *g*). NOD mice also have the most functional activating *Ly49* found to date in one haplotype (four: *Ly49d*, *m*, *p*, and *w*), even though a *Ly49h/u*-like gene has not been identified in this strain. Whether or not autoimmunity can be linked in this diabetes-prone mouse strain to specific activating Ly49, as it has for activating KIR, remains to be seen [3,4]. In this respect it is noteworthy that introducing the NKC haplotype of B6 mice, which has less activating Ly49, significantly reduces the severity of autoimmune disease in these mice [37]. In that regard, it is tempting to speculate that a link may exist between characteristic immune function (for example, Th2 bias) in BALB/c mice and the lack of activating Ly49 in this strain.

Like the NOD-NKC congenic mouse, intra-NKC-recombinant or NKC-congenic mouse strains made from B6 and BALB/c mice have helped to identify the location of regulatory markers in infectious diseases such as MCMV, HSV, and murine malaria [38–40]. The large differences in *Ly49* gene content between BALB/c and B6 mice shown in this report are one reason for their success. Whether any haplotype-specific NK cell receptors are implicated in other diseases, in addition to MCMV, is an important question.

Materials and methods

Identification of BALB/c BACs containing Ly49 genes

High-density spotted filters of the BALB/c genomic library CHORI-28 segment II were purchased from BACPAC Resources (Oakland, CA, USA). The CHORI-28 library was generated by *Eco*RI partial digestion and cloned into the pTARBAC2.1 vector. Library filters were hybridized with radioactively labeled 129 strain *Ly49v*, *s*, *g*, *e*, *o*, *t*, *r*, *u*, *p*, and B6 *Ly49c* cDNAs. Glycerol stocks of positive clones were purchased from BACPAC Resources, and BAC DNA from overnight culture in LB plus 12.5 μg/ml chloramphenicol was isolated using conventional plasmid preparation kits (Qiagen, Mississauga, ON, Canada).

BAC Ly49 gene screening by PCR

Gene content on *Ly49*-positive BACs was determined by PCR and sequencing of products. Briefly, approximately 10 ng of BAC DNA was subjected to PCR (94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min, 30 cycles) with primers capable of amplifying known and predicted BALB/c *Ly49* sequences across exons 3 and 4 (primers are shown in Table 3). PCR products were separated on 1% agarose gels and visualized with ethidium bromide. PCR products of each size were cloned by TOPO TA-cloning (Invitrogen, Montreal, QC, Canada) into pCR2.1 and sequenced in-house using T7 and M13-reverse primers. Partial exon 3 and 4 sequence allowed

Table 3
Primers used to amplify *Ly49* from BAC clones and for detection of full-length cDNA

Ly49	Sense primer (exon 3)	Antisense primer (exon 4)
a	5′-GTTCATTGTGATAGCTCTTGGC	5′-GTTGAGGGATTCCAGAAGATC
c	5′-AGTGTTCAGCACCCTGGCAA	5′-TACTGTCCCATCTGTCCTGT
e	5′-CCCTGGCAGCTCACTGTGAG	5′-GAAGTTCCTCACCTGGACTG
g	5′-GAAGCTCATTGTGATAGCTTG	5′-TGTGTGCTGTGAGGAATCTG
i	5′-GTGTTCAGTATCCTGGCAAC	5′-CCTGTTCTCTTTTGATGTATTCCAGAAT
l	5′-TCCCTGGCAGCTCATTGTGA	5′-CTGTTCCTTGTGGAGGGATT
q	5′-GTGCTCTATCCCCTGGCATC	5′-CCTGTACTACACTCTAGAGG
x	5′-GTGTTCAGTCCCCTGGAAGT (5′ UTR)	5′-CTTGGTTTTATTATACAATCTGTTCTT (3′ UTR)
e	5′-ATACTTCATGCACTCCCACG	5′-TCTGTCTCCAAGAGGAAGG
q	5′-GAACACACTCTACATACACC	5′-CTCTTCCAGCTCTTCCTTCT

the identification of the *Ly49* gene that was amplified from each BAC clone.

RT-PCR detection of BALB/c NK cell Ly49

Full-length cDNA clones were obtained from total RNA isolated from day 6 BALB/c IL-2 activated plastic-adherent NK cells using TRIzol (Invitrogen) and reverse transcribed into cDNA with Superscript First Strand cDNA synthesis kit (Invitrogen). PCR conditions, visualization, cloning, and sequencing were the same as above. All cDNA sequences were determined from at least two separate PCRs. Sequences were deposited with GenBank under the following accession numbers: $Ly49q^{BALB}$ (AY620246), $Ly49e^{BALB}$ (AY620247), $Ly49i^{BALB}$ exon 3 (AY620248), $Ly49i^{BALB}$ exon 4 (AY620249), $Ly49x^{BALB}$ exon 3 (AY620250), and $Ly49x^{BALB}$ exon 4 (AY620251).

BAC Ly49 gene screening by Southern

BAC DNA from a 1-ml overnight culture was isolated using standard techniques for plasmid isolation. Briefly, DNA was digested with *Eco*RI and separated on a 1% agarose, 1× TAE gel. Digestion was confirmed postseparation by ethidium bromide staining, followed by denaturation and neutralization and capillary transfer onto Hybond nylon membrane (Amersham, Montreal, QC, Canada). The membrane was UV cross-linked and then hybridized at 65°C with radioactively labeled exon 3–intron 3–exon 4 PCR fragments cloned and sequence confirmed as described above. Following overnight hybridization, the membrane was washed twice with 65°C-preheated 2× SSC/1% SDS or, to reduce cross-hybridization to closely related *Ly49* genes, the membrane was washed at 70°C in 0.5× SSC/1% SDS for 1 h. The membrane was then exposed to film for various times.

Phylogenetic analysis

Nucleotide and protein sequences were aligned using ClustalX v1.81 [41]. PHYLIP v3.5c was used to assess *Ly49* exon 3–4 similarities between B6 and BALB/c alleles [42]. Briefly, after alignment, a bootstrap dataset of 1000 was created using SEQBOOT, followed by creation of a distance matrix for this dataset with DNADIST, and NEIGHBOR was used to construct trees. Trees were visualized with TreeExplorer 2.12.

BAC sizing

BAC DNA from a 1-ml culture was digested with *Not*I overnight and separated on a 1% agarose, 1× TAE gel using a CHEF-DR III Variable Angle system (Bio-Rad, Mississauga, ON, Canada) with settings of 6 V and a switch time gradient of 0.5 to 54 s over 20 h. The gel was then visualized with ethidium bromide and the BAC fragment sizes were estimated by comparison to a λ concatemer size standard (New England Biolabs, Mississauga, ON, Canada). BAC clone sizes were estimated from four different PFGE experiments of varying separations and averaged.

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