Genetic and expression analyses of the *Nkrp1-Clr* gene cluster

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Abstract

Natural killer (NK) cells, lymphocytes of the innate immune system, can recognize a wide array of cells via several receptors families such as Ly49 and NKR-P1. The *Nkrp1* gene family encode for C-type lectin-like receptors which can recognize their ligands, Clr, on target cells. *Nkrp1* and *Clr* genes are intertwined in the NK gene complex and are thus inherited together. The *Nkrp1-Clr* genes in 129S6 and BALB/c mouse strains show significant sequence polymorphism compared to those of C57BL/6 mice while the overall gene organization and gene number are conserved. RT-PCR was utilized to study the expression of individual *Nkrp1-Clr* genes. *In situ* hybridization was performed to validate expression results from RT-PCR, as well as to verify the cell types in which *Nkrp1-Clr* genes are expressed. Surprisingly, our expression studies reveal an interesting pattern of expression of *Nkrp1* and *Clr* genes not only in lymphoid tissues but also in the epithelial cells of the intestine, kidney, eye and lung, the myocytes of the heart and skeletal muscle, and possibly some endothelial cells, indicating novel functions of NK cells in these tissues.

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Table of contents

List of figures

List of tables

List of abbreviations

aCGH	array-comparative genomic hybridization
ADCC	antibody-dependent cellular cytotoxicity
ALAK	adherent lymphokine activated killer
AP	alkaline phosphatase
β2m	beta-2-microglobulin
B6	C57Bl/6
BAC	bacterial artificial chromosome
BID	BH3-interacting domain death agonist
BSA	bovine serum albumin
CD	cluster of differentiation
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CK2	casein kinase 2
Clr	C-type lectin related
CRD	carbohydrate recognition domain
DAP10/12	DNAX-activating protein of 10/12kDa
DC	dendritic cell
DIG	digoxigenin
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ERV	endogenous retrovirus
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum

Fc	fragment, crystallizable
FITC	fluorescein isothiocyanate
Flt3L	fms-like tyrosine kinase 3 ligand
GM-CSF	granulocyte-macrophage colony-stimulating factor
H&E	hematoxylin and eosin
HCMV	human cytomegalovirus
HLA	human leukocyte antigen
HSC	hematopoietic stem cells
IEC	intestinal epithelial cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRF	interferon-regulatory factor
ITAM	immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KIR	killer immunoglobulin-like receptors
LFA-1	lymphocyte function-associated antigen-1
LINE1	long interspersed nuclear element 1
LLT1	lectin-like transcript 1
LRC	leukocyte receptor complex
LTR	long terminal repeats
mAb	monoclonal antibody
MCMV	murine cytomegalovirus
MHC	major histocompatibility complex
MHC-I	major histocompatibility complex class I

MICA/B	MHC class I polypeptide-related sequence A/B
MIP	macrophage-inflammatory protein
MULT1	murine UL16-binding protein-like transcript 1
NBT/BCIP	nitro blue tetrazolium /5-bromo, 4-chloro, 3-indoylphosphate
NCR	natural cytotoxicity receptor
NK	natural killer
NKC	natural killer complex
NKP	natural killer cell precursors
NOD	non-obese diabetic
PBS	phosphate buffered saline
PIP	percent identity plot
Rae-1	retinoic acid early inducible-1
RCMV	rat cytomegalovirus
RFLP	restriction fragment length polymorphisms
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SCF	stem cell factor
SDS	sodium dodecyl sulfate
SHIP	SH2-containing inositol polyphosphate 5-phosphatase
SHP-1/2	SH2-containing protein tyrosine phosphatase-1/2
SINE	short interspersed element
TNF	tumour necrosis factor
ULBP	UL-16 binding protein
ZAP70	zeta-chain-associated protein kinase 70

1 Introduction

1.1 Natural killer cell history

Natural killer (NK) cells were first discovered in the 1970's when it was observed that lymphocytes, isolated from individuals whom were not previously immunized, had the ability to kill tumor cells (1). Lymphocytes from wildtype mice showed natural cytotoxicity against syngeneic and allogeneic tumors. This cytotoxic effect was seen in the lymph node, spleen, bone marrow and peripheral blood of athymic nude mice which were depleted of T cells (2). Further study of morphology and phenotype showed that the "natural" killer cells represent a distinct group of lymphocytes that differ from lymphocytes with antigen specific receptors due to a lack of the expression of T or B cell markers such as TCR, CD3 and immunoglobulin (Ig) (3, 4). NK cells are characterized as large granular lymphocytes of the innate immune system with the ability to spontaneously kill a wide range of target cells such as virally-infected cells or tumor cells without prior sensitization. NK cells constitute less than 15% of the peripheral blood lymphocyte population (5).

1.2 "Missing-self" hypothesis

NK cells contribute to the first line of defense against virally-infected or transformed cells (6). They are able to distinguish these abnormal cells from healthy cells by surveilling for surface expression of various molecules such as self MHC class I (MHC-I). As proposed by the "missing-self hypothesis", NK cells recognize and kill cells which exhibit abnormal self MHC-I expression, which often occurs during virus infection or tumor transformation (7, 8). If the ligands for both the activating and inhibitory receptor are absent or if only the ligands for the inhibitory receptors are present on the cell surface, NK cells will spare these target cells. If only the activating receptors are expressed on the target cells and MHC-I ligands for the inhibitory receptors are absent, NK cells will kill these targets cells. If ligands

for both the inhibitory and activating NK receptors are expressed on the target cells, whether the NK cell will kill these cells or not depends on the integration of signals originating from the activating and inhibitory receptors (6). That is, if the activating signal dominates, NK cells will kill the target cells, while if the inhibitory signal dominates, NK cells will spare the target cells. Virally-infected cells or tumor cells tend to downregulate expression of MHC-I in order to avoid recognition and killing by cytotoxic T cells (9, 10). This downregulation however makes them susceptible to recognition by NK cells, which can lead to NK cell-mediated killing. Interestingly, human erythrocytes without surface expression of MHC-I and neuronal cells with low MHC-I expression are spared from killing by NK cells (6). The explanation could be that there are insufficient ligands expressed on the cell surface to trigger activating receptors (11-13). Alternatively, there may be ligands other than MHC-I that engage inhibitory receptors on NK cells and dampen NK cell activation, such as Clr-b and CD48, which are recognized by mouse inhibitory NKR-P1B and 2B4, respectively (6, 14, 15).

1.3 NK cell education

Interactions between inhibitory NK cell receptors and self MHC-I molecules are important for NK cell education (16-18). NK cells from $\beta_2m^{-/-}$ mice which lack MHC-I expression cannot recognize MHC-I deficient cells. These NK cells are generally considered as hyporesponsive due to their defective recognition of "missing-self" cells (19). H-2D^d transgenic B6 mice can recognize H-2D^d in addition to self MHC H-2^b in wildtype B6 mice, thus cells from wildtype B6 are considered as loss of H-2D^d expression and thus are rejected by NK cells from H-2D^d transgenic B6 mice (20). Therefore, NK cell education depends on the expression of inhibitory receptors that bind to self MHC-I molecules during NK cell

development. Several models exist to explain NK cell education. The arming model suggests that NK cells are originally anergic and those that have inhibitory receptors for self MHC-I will acquire functionality while those that lack self MHC-specific inhibitory receptors will become hyporesponsive (16). In the disarming model, it states that NK cells are originally responsive. They become disarmed if they do not have inhibitory receptors for self MHC-I to maintain self-tolerance and thus are anergic or hyporesponsive; on the other hand, if they do express inhibitory receptors they will become responsive (21). More recently, the rheostat model was proposed, stating that NK responsiveness depends on the strength of inhibitory signals received by NK cells and that NK cells change their activation threshold in response to their environment. Thus, NK cells keep themselves in a well-tuned state to achieve self-tolerance as well as optimal activation (22). Lastly, as MHC-I molecules are expressed on all nucleated cells including NK cells, it is possible that inhibitory receptors on NK cells will bind to MHC-I on the same cell. This *cis* interaction results in fewer inhibitory receptors available to engage target cells, thus lower inhibitory signals will be generated and NK cells become more responsive against target cells (23).

1.4 NK cell development

Bone marrow is considered as the main site for NK cell development as ablation of bone marrow results in failure to generate NK cells in mice (24). NK cells develop from hematopoietic stem cells (HSC) which then undergo serial development stages and become more lineage restricted NK cells precursors (NKP) (25). NKPs are found in the liver, spleen, lymph node and thymus, suggesting that these tissues may be sites for NK cell development as well (26). There are five stages in murine NK cell development within the bone marrow, which can be distinguished by the surface makers expressed by the cell (27). At stage I,

NKPs express IL-2/15Rβ (CD122) and they lack the ability to differentiate into T cells or B cells. As NKPs develop, they gradually acquire the expression of NK cell receptors. At stage II, immature NK cells begin to express NK1.1 and CD94/NKG2. They also express low levels of DX5, CD11b and CD43. Stage III cells start to express Ly49 and c-Kit. Stage IV is the expansion stage in which cells exhibit increased expression of DX5, and start to gain low cytotoxic ability and cytokine secretion. Once these cells enter stage V, they are considered to be mature. They have lost expression of c-Kit but have higher expression of CD11b and CD43. In addition, these mature NK cells are highly cytotoxic and can secrete cytokines (27).

IL-15 has been shown to be important for NK cell development, proliferation and survival of peripheral NK cells. Mice deficient in IL-15 lack NK cells (28). Cytokines such as FMS-like tyrosine kinase 3 ligand (FLT3L) and stem cell factor (SCF) can facilitate the generation of NKP, and it has been shown that *FLT3L*$^{-/-}$ mice have a lower number of NK cells (29). Transcription factors have also been shown to play an important role in NK cell development. Ets-1, Id2, Ikaros, PU.1 are essential for generating NKP while Gata-3, IRF-2 and T-bet are important for NK cell maturation (30).

1.5 Distribution of NK cells

Due to the protective role of NK cells in the innate immune system, their distribution in both lymphoid and non-lymphoid tissues is localized to areas in which pathogens enter the host. They are found in a decreasing frequency as follows: lung, liver, peripheral blood, spleen, bone marrow, lymph node and thymus (31). NK cells can be found in the intestine and placenta as well. In the spleen, NK cells are predominantly found in the red pulp area and a few NK cells have been detected in the marginal zone, but not white pulp (31). Within the lymph node, NK cells are present in the perifollicular area, paracortex and medullar zone

(32).

1.6 Effector functions of NK cells

Upon encountering and recognizing their target cells, activated NK cells will release effector molecules like granzymes and perforins to initiate cytotoxicity without transcription or proliferation (6). The exact mechanisms of how perforin works remains unknown, but it is known that they are able to disrupt the membrane of target cells. It was thought that perforin can create pore-like structures on the membrane of target cells so that granzymes can enter (33); however, it was later discovered that granzymes B can be internalized into the target cells independently of perforin (34, 35). Granzyme B was shown to mediate apoptosis in target cells via caspase cascade or through BH3-interacting domain death agonist (BID) that can cause the release of cytochrome *c* from mitochondria (36-39). NK cells are also able to mediate cytotoxicity via the binding of the Fas ligand on NK cells and Fas on target cells (40).

NK cells secrete cytokines and chemokines to shape the immune response. This function of NK cells will induce adaptive immunity and thus NK cells act as an important link between innate and adaptive immunity. Once activated, NK cells tend to produce IFN-γ which aids in boosting the immune response. NK cells also secrete cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α, interleukin 10 (IL-10) and chemokines such as macrophage inflammatory protein 1 (MIP-1) that can recruit other immune cells (5, 41). IL-10 is able to inhibit the immune response of macrophages and NK cells (42). NK cells interact with dendritic cells (DCs) by killing immature DCs, as well as secreting cytokines such as IFN-γ and TNF-α to promote the maturation of DCs to fulfill their role as antigen presenters to T cells (14, 43). Interestingly,

at high DC:NK ratio the activation of DCs is dominant while low DC:NK ratio leads to the killing of immature DCs by NK cells (44). NK cells express co-stimulatory ligands to activate T and B cells (45) and can also kill activated $CD4^+$ T cells and over-activated macrophages (14, 46, 47).

1.7 Subsets of NK cells

Human NK cells are $CD3^-$ and are divided into two subsets according to the surface expression intensity of CD56 and CD16: $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$ (48). Human NK cells develop from common lymphoid progenitor cells, which then generate pro-NK, pre-NK, immature NK, $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$ NK cell types (49). $CD56^{bright}CD16^-$ NK cells are mostly found in secondary lymphoid tissues such as the lymph nodes and tonsils. They usually have dampened cytotoxicity and a strong ability to secrete cytokines. In comparison, $CD56^{dim}CD16^+$ NK cells are primarily found in the bone marrow, circulating blood and spleen. These NK cells are armed with the ability to lyse target cells; however, they are compromised in terms of cytokine secretion (48, 50). $CD56^{bright}CD16^-$ NK cell subsets have constitutive surface expression of IL-2R and the c-kit receptor which allows them to proliferate quickly in response to IL-2 treatment (51-54). *In vitro* activation of $CD56^{bright}CD16^-$ NK cell subset with IL-2 makes $CD56^{bright}CD16^-$ NK cells and $CD56^{dim}CD16^+$ NK cells acquire almost the same level of cytotoxicity (55, 56). Human NK cells also express other receptors such as CD94/NKG2A, NKG2D and natural cytotoxicity receptors (NCRs), and as such, they are able to recognize abnormal cells (48).

Murine NK cells undergo three development stages: $CD27^+CD11b^{dull}$ to $CD27^+CD11b^+$ and to the mature $CD27^{dull}CD11b^+$ (14). $CD27^{dull}CD11b^+$ NK cells have high cytotoxicity while $CD27^+CD11b^{dull}$ and $CD27^+CD11b^+$ NK cells are more capable of producing cytokines

(57). $CD27^{+}CD11b^{dull}$ NK cells are mainly found in the bone marrow and lymph nodes where NK cells develop. $CD27^{dull}CD11b^{+}$ NK cells are dominant in the peripheral blood, spleen, liver and lung (14, 58). In order to recognize aberrant cells, the mouse and human NK cells share common receptors such as NKG2D and CD94/NKG2; in other cases, they express homologous receptors such as Ly49 and KIRs (59).

1.8 NK cell receptors

In mice, NK cells recognize a wide array of cells via several receptors expressed on their surface, including Ly49, NKR-P1, CD94, NKG2A/C/E and NKG2D. This expansive receptor repertoire allows NK cells to tolerate self cells and lyse abnormal cells. The decision of whether NK cells kill a target cell depends on the integration of signals originating from different receptors (6, 60).

Inhibitory NK cell receptors have one or more copies of the immune-receptor tyrosine based inhibitory motif (ITIM) in their cytoplasmic domain. The ITIM consensus motif consists of Ile/Val/Leu/Ser-x-Tyr-x-x-Leu/Val (6). Upon ligand binding, the tyrosine residues of the ITIM will become phosphorylated by Src family kinases and recruit tyrosine phosphotases like SHP (Src homology region 2-containing protein-tyrosine phosphatase)-1, SHP-2 or phospholipid-specific phosphatase SHIP, which will lead to the dephosphorylation of downstream signaling molecules responsible for NK cell activation including FcεRIγ, ZAP70, Syk and Vav-1. As a result, NK cell activity is inhibited (6, 61).

In contrast, activating NK cell receptors have short cytoplasmic domains and do not possess signal transduction motifs for stimulating NK cells. However, they are positively charged in their transmembrane domain, and this allows for their association with adaptor proteins like DAP12, FcRγ and CD3ζ, which have an immune-receptor tyrosine based

activating motif (ITAM) in their cytoplasmic domains characterized by the following consensus sequence: Asp/Glu-x-x-Tyr-x-x-Leu/Ile-x_{6-8}-Tyr-x-x-Leu/Ile (6). Upon binding to their ligands, tyrosine residues within the ITAM of the adaptor protein will be phosphorylated, thus kinases like Syk or ZAP70 are recruited, resulting in phosphorylation of downstream signal molecules and leading to the activation of NK cells (6, 61). Another adaptor protein, DAP10, can associate with the activating receptors as well; it is characterized by the consensus sequence Tyr-x-x-Met, and can deliver activating signals independent of the Syk protein kinase family (62).

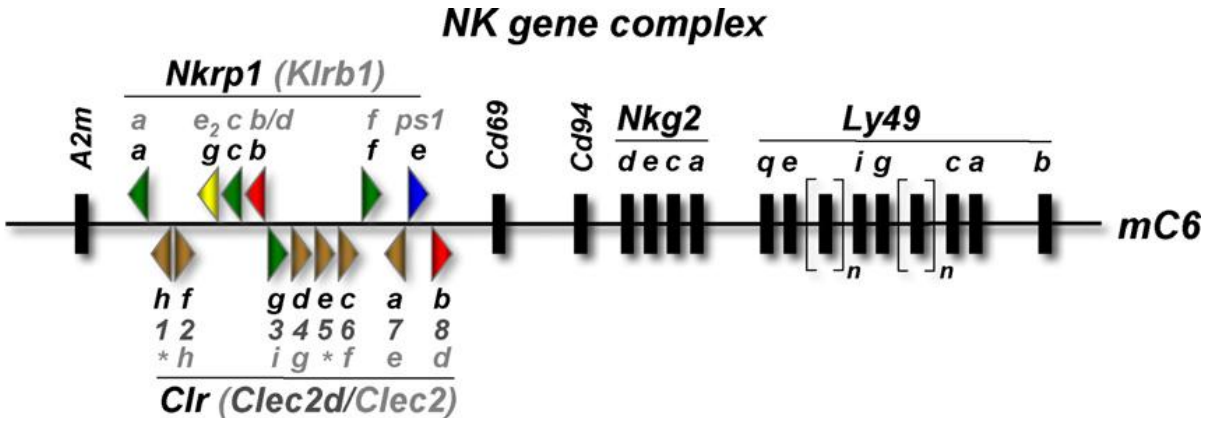
The balance of signals originating from the activating and inhibitory receptors determines whether NK cells will be stimulated. Specifically, if the activating signal dominates, NK cells will lyse target cells by direct cytotoxicity via granzymes and perforins, or boost the immune response by secreting cytokines like IFN-γ, TNF-α and chemokines. However, if the inhibitory signal dominates, NK cells will not be activated and will spare these target cells (6).

NK receptors fall into two categories: immunoglobulin superfamily and C-type lectin like receptor family. NK cell receptors are encoded by genes that are present mainly in two domains: leukocyte receptor complex (LRC) and NK gene complex (NKC) (**Figure 1**). Receptors that are encoded within the LRC belong to the immunoglobulin superfamily while receptors encoded by genes present in the NKC belong to C-type lectin like family (63, 64).

1.8.1 CD16

CD16 (FcγRIII) was the first activating receptor studied on NK cells. It has low binding affinity to the Fc portion of antibodies and mediates antibody dependent cellular cytotoxicity (ADCC) to kill antibody-coated pathogens (60, 65). CD16 is a transmembrane protein that

Figure 1: Map of the mouse NK gene complex (NKC). The map shows the relative gene organization within the NKC from the centromeric end to the telomeric end (left to right) on mouse chromosome 6.
Reprinted from Seminars in Immunology, 20 (6), Carlyle J.R, Mesci A, Fine J.H, Chen P, Bédanger S, Tai L.H, Makrigiannis A.P., Evolution of the Ly49 and Nkrp1 recognition systems, 321-330, Copyright (2008), with permission from Elsevier.


NK gene complex
Nkrp1 (Klrb1)
A2m
a
a
e2 c b/d
g c b
f ps1
f e
Cd69
Cd94
Nkg2
d e c a
Ly49
q e
i g
c a
b
mC6
n
n
h f
1 2
* h
g d e c
3 4 5 6
i g * f
a b
7 8
e d
Clr (Clec2d/Clec2)

belongs to the immunoglobulin superfamily (66). It associates with the adaptor protein FcεRIγ in mice and FcεRIγ or CD3ζ in humans (67-69). Upon engagement with its ligand, members of the Src kinase family will phosphorylate the tyrosine residue in the ITAM motif within the adaptor protein (70, 71). This leads to the recruitment of tyrosine kinases such as ZAP70 or Syk and the phosphorylation of downstream substrates (72), resulting in NK cell activation which includes Ca^{2+} mobilization, degranulation, transcription and secretion of cytokines and chemokines (6).

1.8.2 CD94/NKG2

CD94/NKG2 heterodimer receptors belong to the C-type lectin-like receptor family (73). They are expressed on NK cells as well as a small population of T cells including γδT cells and effector $CD8^{+}$αβ T cells (74, 75). Several NKG2 molecules were found to dimerize with CD94 including NKG2A, NKG2C and NKG2E (73). NKG2A, NKG2C and NKG2E have diverse extracellular and intracellular domains, suggesting a difference between these receptors in ligand recognition and signal transmission. Interestingly, NKG2A is characterized as an inhibitory receptor and has an ITIM domain in its cytoplasmic domain (76, 77). In contrast, NKG2C and NKG2E lack the ITIM and thus associate with adaptor proteins to deliver activating signals (73, 78). The CD94/NKG2 receptor binds to $Qa\text{-}1^{b}$ molecules in mice and HLA-E in humans, both of which are non-classical MHC-Ib molecules and can present the leader sequence of other MHC-I molecules (79-81). The affinity of the activating CD94/NKG2C and CD94/NKG2E for HLA-E is much lower than for the inhibitory CD94/NKG2A, suggesting that the interaction of CD94/NKG2C and CD94/NKG2E with HLA-E will not activate NK cells under normal circumstances (82).

CD94/NKG2 receptors display low polymorphism, which might be due to the limited polymorphism of their ligands (6, 81).

1.8.3 NKG2D

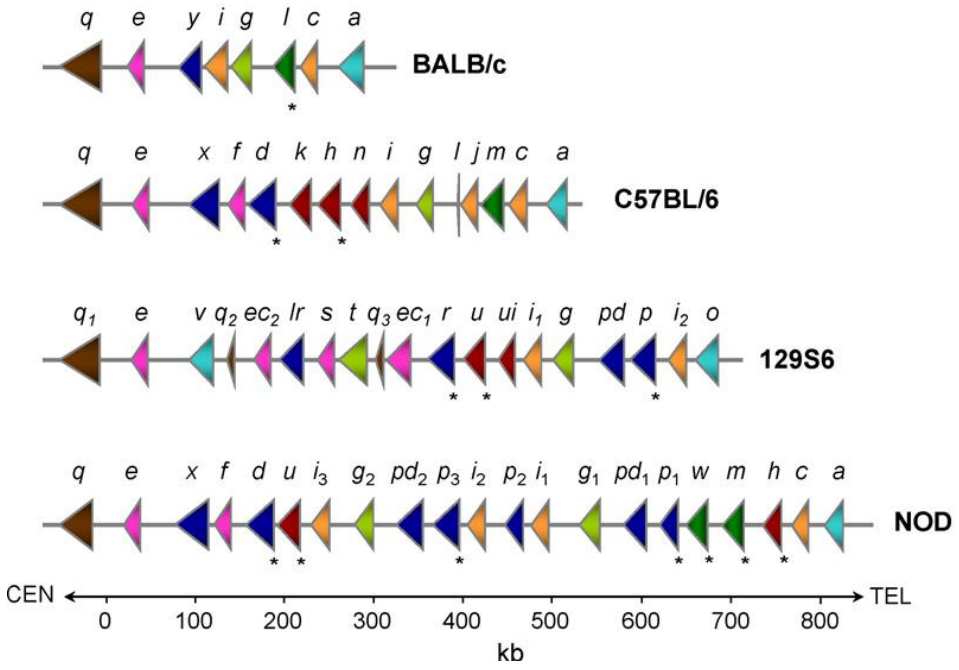
The NKG2D receptor is a type II transmembrane protein that also belongs to the C-type lectin family. Unlike other NKG2 which form a heterodimer with CD94, the NKG2D receptor is a homodimer and associates with adaptor proteins for its surface expression (83). NKG2D can recognize MHC-I homologs, including transmembrane proteins such as human MICA/B, ULBP4 and mouse H60, MULT-1, as well as phosphatidylinositol glycan anchored proteins such as human ULBP1-3 and mouse RAE-1 (6). Interestingly, NKG2D ligands are expressed at low levels on the surface of normal healthy cells but are induced and highly expressed on the cell surface under stress conditions such as viral infection (84). When engaged with its ligand, the ITAM within the intracellular domain of the adaptor protein DAP10 or DAP12 becomes phosphorylated and stimulatory signals are transmitted, leading to NK cell activation (83).

1.8.4 Ly49

Ly49 receptors belong to the C-type lectin-like family and are type II transmembrane proteins. The ligands for Ly49 are MHC-I molecules. Most of the Ly49 receptors are inhibitory and possess ITIMs in their cytoplasmic domain (6). Though Ly49 receptors are predominantly classified as being inhibitory, there are some activating receptors as well, such as Ly49D and Ly49H in B6 mice. These activating receptors have positively charged arginine residues in their transmembrane domain, which allows for their association with negatively-charged residue in the ITAM-containing adaptor proteins such as DAP12 (85). ITAM containing adaptor proteins stimulate NK cell function upon the engagement of

activating Ly49 receptors and their ligands. These adaptor proteins are vital for the surface expression of activating Ly49 receptors (85, 86). Interaction of Ly49 with MHC-I molecules inhibit NK cell activation; however, when expression of MHC-I is absent on target cells, NK cells will become activated. NK cells express only a fraction of the Ly49 receptors, ranging from zero to six, though more commonly expressing two to three (87). This creates a diverse repertoire of NK cells that can surveil small changes in MHC-I isoform expression. *Ly49* gene family are found to have diverse gene content and significantly high gene allelic polymorphism among different inbred mouse strains such as B6, 129S6, BALB/c and NOD (**Figure 2**) (88-91). This may be due to the significantly high percentage of LINE1 elements in the *Ly49* gene cluster compared to the overall percentage in the whole genome (92). Homologous recombination between different Ly49 receptors may lead to the formation of completely new Ly49 receptors comprised of the ligand binding domain from one Ly49 receptor and intracellular signaling domain from another Ly49 receptor. This indicates the possibility for the new Ly49 receptor to maintain the ligand binding affinity while altering the signaling property between inhibitory and activating (6, 92). This evolutionary strategy may have an important role in host recognition of viral mimics of self-ligands (11). Ly49H is responsible for the resistance of B6 mice to murine cytomegalovirus (MCMV) infection (93). Ly49H has been shown to have high affinity with the m157 viral glycoprotein. m157 has high homology with MHC-I and can bind to inhibitory Ly49 receptors such as Ly49I. It is thus speculated that during evolution, MCMV has evolved m157 to mimic self MHC-I in order to evade host NK cell killing. In response to this, the host may have evolved Ly49H through gene conversion by specifically combining the extracellular virus recognition domain of the inhibitory receptor Ly49I and the activating intracellular signaling domain of

Figure 2: Comparison of the *Ly49* haplotypes among different inbred mouse strains. The genomic organization of *Ly49* genes from BALB/c, C57BL/6, 129S6 and NOD are represented. The gene contents are highly polymorphic among different mouse strains. Reprinted from Seminars in Immunology, 20 (6), Carlyle J.R, Mesci A, Fine J.H, Chen P, Bédanger S, Tai L.H, Makrigiannis A.P., Evolution of the Ly49 and Nkrp1 recognition systems, 321-330, Copyright (2008), with permission from Elsevier.



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

another activating receptor. Ly49H, with high affinity binding m157, can thus lead to NK cell activation and killing of MCMV infected cells. Meanwhile, Ly49H lost the ability to bind to self MHC-I molecules to avoid autoimmunity (11). Both *cis* and *trans* interactions between Ly49 and MHC-I was found. *Trans* interaction means that Ly49 on NK cells engage with MHC-I molecules expressed on target cells while *cis* interaction means that Ly49 and MHC-I expressed on the same NK cells interact. *Cis* interaction between inhibitory Ly49 with MHC-I molecules is thought to play a role in decreasing the threshold to activate NK cells (94, 95).

1.8.5 KIR

The human homologue of Ly49 is the killer immunoglobulin-like receptors (KIR). *KIR* genes are expressed on NK cells, γδ T cells and effector T cells, mainly $CD8^+$ T cells (6). Unlike the Ly49 family which have a C-type lectin-like structure, KIRs are type I transmembrane glycoproteins with two or three Ig-like extracellular domains. The KIR family can be divided into two subfamilies: KIR3D and KIR2D (96-98). Although KIR and Ly49 differ in their structure, they share similar signaling pathways for regulating NK cell function (6). The KIR family members have both activating and inhibitory receptors that modulate NK cell function. The length of the cytoplasmic domain of KIR3D and KIR2D define their signaling characteristics. KIRs with long intracellular domains have one or two ITIMs which make them potential inhibitory receptors. On the other hand, KIRs with short intracellular domain do not have ITIM sequences; rather, they have charged residues in their transmembrane domain which make them candidates for stimulating NK cells (99). The ligands for KIRs include the HLA-A, HLA-B and HLA-C proteins. The activating KIR receptor has low or no affinity with self HLA molecules, which might have been selected for

in order to avoid autoimmunity (6). *KIR* genes, and the genes encoding for their ligands, HLA, are highly polymorphic (99).

1.9 NKR-P1 receptors

1.9.1 NKR-P1 receptor structure

NKR-P1 receptors are C-type lectin-like disulfide-linked type II transmembrane proteins that are encoded by *Nkrp1* genes close to the centromeric domain of the NKC on mouse chromosome 6, human chromosome 12 and rat chromosome 4 (6). The first NKR-P1 receptor identified was NKR-P1C, also known as NK1.1. It has long been used as a marker to define NK cells in certain mouse strains (100). The mouse NKR-P1 receptor family includes several members, namely NKR-P1A, NKR-P1B/D, NKR-P1C, NKR-P1E, NKR-P1F and NKR-P1G, among which *Nkrp1e* appears to be a pseudogene due to many stop codons present in its open reading frame (92). NKR-P1B and NKR-P1D are alleles of the same gene. NKR-P1C has a positively-charged residue in its transmembrane domain which allows for its association with the negatively-charged residue in the ITAM-containing adaptor protein FcRγ. When cross-linked via mAb, NKR-P1C delivers an activating signal (101, 102). NKR-P1A and NKR-P1F are found to have positively-charged residues present in their transmembrane domain as well, indicating that these two proteins may function as activating receptors (6). NKR-P1B and NKR-P1G do not possess a positive charge in their transmembrane domain; rather, they have ITIMs in the cytoplasmic tail that can inhibit the NK cell activation cascade upon phosphorylation (103).

1.9.2 NKR-P1 receptor ligands

The *Nkrp1* genes are composed of six exons. The first exon encodes for the N-terminal portion of the protein which is the cytoplasmic domain. The second exon encodes for the

transmembrane domain. The third exon encodes for the variable stalk domain. Exons four through six encode for the extracellular domain which is the carbohydrate recognition domain (CRD) that will recognize the ligand (104, 105). However, the extracellular CRD domain does not provide these NKR-P1 receptors with the ability to functionally bind to carbohydrates due to the lack of calcium binding domains which are essential for authentic carbohydrate recognition (106). Physiological ligands for NKR-P1 were not identified until 2003 and were found to be C-type lectin-like proteins themselves (107). These ligands are encoded by *Clr* genes that are intertwined with *Nkrp1* genes within the NKC and inherited together (108). Several members belong to the Clr receptor family namely: Clr-a, -b, -c, -d, -e, -f, -g, -h, -i, -j, although the functionality of some members is unknown (109). NKR-P1B/D recognizes Clr-b and results in inhibition of NK cells, while NKR-P1F recognizes Clr-g and causes stimulation of NK cells (107, 110). It was then discovered that Clr-c and Clr-d are ligands for mouse NKR-P1F and Clr-f, Clr-g and Clr-d are ligands for mouse NKR-P1G (109, 111). The physiological ligands for the NKR-P1A and NKR-P1C remain unknown. Phylogenetic analysis shows that *Nkrp1a/b/c/e* are clustered together, with *Nkrp1b* being more distant from the closely related *Nkrp1a/c/e*. *Nkrp1f/g* are clustered together and are separate from *Nkrp1a/b/c/e*. As for *Clr* genes, *Clrb/d/g* and *Clra/f* are clustered and segregate from each other while *Clrc* and *Clrh* are more distant (109).

1.9.3 NKR-P1 signal transduction

Ligand engagement of mouse NKR-P1C causes intracellular Ca^{2+} influx, phosphatidylinositol turnover (112), kinase phosphorylation and cytokine secretion (102). However, disruption of the CxCP motif within the cytoplamic domain of NKR-P1C and NKR-P1B receptors, which is responsible for the association of the receptors with the Src

family kinase $p56^{lck}$, inhibits NK cytotoxicity. The important role of Lck in NKR-P1B/C receptor signaling initiation was further confirmed by the failure of anti-NK1.1 induced Ca^{2+} influx in $Lck^{-/-}$ mice. Furthermore, mutation of the ITIM cytoplamic domain in NKR-P1B receptor results in loss of SHP-1 recruitment and inhibitory signal transduction. All the above findings suggest that Lck is important in the initiation of NKR-P1B/C signaling and that the ITIM domain of the inhibitory receptor is essential for inhibitory signal transduction (113).

1.9.4 Human NKR-P1 receptor

In humans, only one inhibitory receptor, NKR-P1A, exists (114) and it binds to lectin-like transcript 1 (LLT1) (115). The *LLT1* gene is closely located next to the *Nkrp1A* gene on human chromosome 12. The engagement of human NKR-P1A receptors on NK cells with LLT1 on target cells results in inhibition of NK cell cytotoxicity and IFN-γ secretion (115). In contrast, NKR-P1A/LLT1 interaction is able to enhance IFN-γ production by T cells in the presence of CD3 (116). LLT1 is broadly expressed and is upregulated upon activation of DCs, B cells, T cells and NK cells (117, 118). The fact that LLT1 is expressed on the surface of NK cells and that upon binding with LLT1 mAb, IFN-γ is produced, sheds light on the role of LLT1 as both a ligand for NKR-P1A in humans but also as a signaling molecule itself (119). The expression of both NKR-P1A and LLT1 on NK cells and T cells raises the possibility of *cis*-interaction (116). Interestingly, LLT1 has a short cytoplasmic domain, therefore, it does not possess the ITIM, instead, it has a charged residue in its transmembrane domain which facilitates its association with adaptor proteins leading to signal transduction (108).

1.9.5 Rat NKR-P1 receptor

In rat BN strain, four NKR-P1 receptors have been identified: NKR-P1A,-P1B,-P1F and -P1G (120). NKR-P1A and NKR-P1F are activating receptors, while NKR-P1B and NKR-P1G are inhibitory receptors (121). There are eleven known Clr proteins, Clr1-11, but Clr8 is a gene fragment (120). Clr9, Clr10 and Clr11 are homologs of mouse Clr-f, Clr-a and Clr-b, respectively (121). The interaction between NKR-P1 and Clr in rats include NKR-P1A: Clr11; NKR-P1B: Clr11; NKR-P1F: Clr2, Clr3, Clr4, Clr6 and Clr7; NKR-P1G: Clr2, Clr6, Clr7 and Clr4 (121).

1.9.6 *Nkrp1* polymorphism

Previous studies show that the related *Ly49* gene family haplotypes are highly polymorphic between different inbred mouse strains. The ligands for Ly49 are MHC-I molecules that are encoded by genes on a different chromosome from that of *Ly49*. This allows for independent inheritance and individual polymorphism of genes encoding for this receptor-ligand pair. However, genes encoding NKR-P1 and its ligand Clr are interspersed within the same chromosome region, thus these two gene families will co-segregate with minimal divergence in order to maintain the interaction between the receptor and its respective ligand (92). Interestingly, comparison of the *Nkrp1-Clr* gene cluster in B6 and BALB/c mouse strains shows that they have the same gene content and genome organization. The relative gene order is as follows, from the centromeric end: *Nkrp1a*, *Clr-h*, *Clr-f*, *Nkrp1g*, *Nkrp1c*, *Nkrp1d*, *Clr-g*, *Clr-d*, *Clr-e*, *Clr-c*, *Nkrp1f*, *Clr-a*, *Nkrp1e* and *Clr-b* (122). Based on a constructed gene map for the *Nkrp1-Clr* gene cluster of 129S6 mice, it was noted that the 129S6 mouse strain has the same genome organization as that of B6 and BALB/c (109). However, the genomic conservation and allelic polymorphism for the *Nkrp1-Clr* gene cluster is not well characterized.

1.9.7 *Nkrp1-Clr* expression

Clr-b is expressed in the thymus, bone marrow, lung, ovary, spleen, ileum of the small intestine, liver, kidney and NK cells, in a broad manner similar to MHC-I molecules. The *Clr-f* transcript is expressed in the ileum of the small intestine, liver, kidney and NK cells. The *Clr-g* transcript was only detected in NK cells (104). Using Northern blot analysis, the *Clr-g* transcript was detected in the spleen and thymus, but not in the brain, heart, liver, kidney, testis, lung, placenta or muscle. RT-PCR for *Clr-g* transcript expression further confirms the expression of *Clr-g* on immune cells including B cells, DCs, as well as activated $CD4^+$ and $CD8^+$ T cells (123). NKR-P1 receptors were predominantly found on NK cells, NKT cells and $CD8^+$ T cells. *Nkrp1f* transcript was found to be highly expressed on DCs and activated $CD8^+$ T cells, suggesting the possible role of NKR-P1F in T cell costimulation (123). A novel population of NKR-P1B^{bright} NK cells were recently found to be predominantly expressed in the blood, liver and gut lymphoid tissues, where NK cells need to be tolerant to self or to dampen activating signals (124). While select *Nkrp1* and *Clr* genes have been studied to date, transcript expression of other *Nkrp1* and *Clr* genes remains to be studied.

Transcript expression for the *Nkrp1-Clr* gene cluster is best characterized in rats. Notably, *Nkrp1a*, *Nkrp1b* and *Nkrp1f* transcripts are highly expressed in NK cells while *Nkrp1g* is moderately expressed. *Nkrp1a* and *Nkrp1b* have also been detected in DC. In addition, high expression of *Nkrp1a* in the spleen and *Nkrp1f* in the testes has been detected. *Clr6* and *Clr7* are highly expressed in the bone marrow. *Clr10* is highly expressed in DC, bone marrow, thymus, macrophages. *Clr11* has a broad expression pattern and high transcript expression has been detected in B cells, NK cells, bone marrow, spleen, thymus, liver, heart, muscle, and kidney (120).

Human NKR-P1A receptor is predominantly expressed on T cells including effector T cells and γδ T cells, and is only expressed in a subset of NK cells (114).

1.9.8 NKR-P1B:Clr-b extended "missing-self" recognition

Clr-b was first recognized as osteoclast inhibitory proteins that play a role in bone formation (125). Clr-b, with a similar expression pattern to MHC-I molecules, is broadly expressed on most hematopoietic cells but not red blood cells (110). Clr-b expression is significantly downregulated after poxvirus infection and this abrogates the inhibitory signal from the engagement of NKR-P1B and Clr-b (126). Clr-b is found to be downregulated on tumor cells as well, which results in NKR-P1B/D recognition of loss of Clr-b expression (110). It was proposed that tumor cells have high expression of casein kinase 2 (CK2) which induces endocytosis of surface Clr-b to avoid phosphorylation by CK2. In addition, Clr-b tends to favor apoptosis of uncontrolled cell growth; as such, the downregulation of Clr-b would be an evolutionary survival strategy used by tumor cells to avoid apoptosis (110). Chemotherapy-induced genotoxic stress induces rapid downregulation of Clr-b at both the RNA and protein level. This loss of Clr-b is recognized by NKR-P1B expressing NK cells and dampens inhibition by the receptor-ligand pair NKR-P1B:Clr-b. In addition, NKG2D ligands are upregulated in stressed cells, which might contribute to the enhanced lytic activity of NK cells; however, MHC-I expression in these stressed cells remains normal (127). Interestingly, expression of Clr-b on the surface of target cells can inhibit killing by NK cells through the inhibitory NKR-P1B/D receptor (107, 110). The MHC-I deficient tumor cell line, RMA-S, is protected from NK cell killing when Clr-b is expressed on the surface (107), suggesting that the NKR-P1B/D and Clr-b receptor-ligand pair represents a new MHC-I-independent NK cell "missing-self" recognition system. Following rat cytomegalovirus

(RCMV) infection, host Clr-b expression is quickly downregulated, rendering the virally infected cell susceptible to NKR-P1B/D mediated “missing-self” recognition. RCMV can induce expression of a Clr-b ortholog on the surface of cells infected with RCMV which functions as a decoy for Clr-b. This interaction with NKR-P1B/D can generate inhibitory signals to dampen or prevent NK cell activation (128). The functional significance of other NKR-P1-Clr receptor-ligand pairs remains unknown.

1.10 Objective

To characterize the *Nkrp1-Clr* gene family conservation and function in different mouse strains; genetic analysis and expression studies were performed. The specific objectives are:

(1) To study the genomic organization and to assess the relative conservation of *Nkrp1-Clr* gene cluster.

(2) To further characterize the allelic polymorphism of individual *Nkrp1-Clr* genes.

(3) To identify the tissues and organs in which *Nkrp1* and *Clr* gene transcripts are expressed in different mouse strains, and to compare the expression pattern of each gene among different tissues across mouse strains.

(4) To specifically identify the cell types where *Nkrp1-Clr* genes were expressed in order to gain further understanding of the potential role of these genes in the tissues in which they are expressed.

2 Materials and Methods

2.1 Mice

B6 and 129S1 mice were purchased from The Jackson Laboratory (Bar Harbour, ME, USA) and BALB/c mice were kindly provided by Dr. Lee-Hwa Tai (Ottawa Hospitals Research Institute, Ontario). Mice were bred and maintained at the Animal Care and Veterinary Services (ACVS) at Roger Guindon Hall (University of Ottawa, Ontario). All manipulations performed on animals were in accordance with university guidelines and approved by the University of Ottawa animal ethics committee.

2.2 cDNA cloning

Total RNA was extracted from adherent IL-2 lymphokine-activated killer (ALAK) cells and fresh intestinal tissues using RiboZol RNA extraction reagent (AMRESCO) following manufacturer's instructions. cDNA was synthesized using verso cDNA kit (Thermo Scientific). Full length *Clr-a* and *Clr-c*/*Clr-d* coding sequences were amplified from intestinal cDNA and ALAK cDNA, respectively, with the primers shown in **Table 1**. PCR products were cloned into the pCR2.1-TOPO vector using TOPO TA cloning kit (Invitrogen). Plasmids were isolated with EZ-10 Spin Column Plasmid DNA Minipreps Kit (Bio Basic Inc.) and sequenced with vector specific primer, T7 promoter.

2.3 Genomic sequence analysis

Various families of repeat elements within the nucleotide sequence were identified with RepeatMasker version 3.3.0 (A.F.A. Smit, R. Hubley & P. Green unpublished; available at http://www.repeatmasker.org), with the following settings: Search engine: Cross_match; Speed/Sensitivity: default; DNA source: mouse. A Percent Identity Plot (PIP) of the repeat-masked sequence of B6, 129S6 and BALB/c *Nkrp1-Clr* cluster sequence compared to B6

Table 1: Primers used to amplify *Nkrp1-Clr* genes from tissue cDNA and plasmids

Primer specificity	Sense primer	Antisense primer
Clr-a	CAAAGGTTGAAGAGGCTTCC	TCACGCATGCTTTGGCACAT
Clr-b	ACTCAGCTCCTCAGCTCTGA	GGCTAAAAAGCGTCTCTTGG
Clr-c	GTTATGACAGCCTCACAGGA	GCTAGCACTGTAACATATAG
Clr-d	GTTATGAATGCACAGTGCCT	GCTAGACAGGAACAGGAGTT
Clr-f	TTGAAACGAGTTCCATGGGC	GGTCATAGAGCATCTGATTG
Clr-g	AGATTGCTTGGAGACAGGAG	GAAGAGTCTCTTGGTAAGTG
Clr-h	GTTATGCAGATGAAGGCGAA	GCTAGCACAGTAAGGTGTAG
Nkrp1a	TGATGCATCTCCTATGCACA	CTCTTGGACAGGATCTGAGT
Nkrp1d^{B6}	GGTGTCAAGTCCCTCCATCT	CATCAGAATTGAAAGCTGTG
Nkrp1b$^{129\&BALB}$	ATGGATTCAACAACACTGGT	TCAGGAGTCATTACACGGGG
Nkrp1c	CAGTGGATCCCCATCAAGAGA AAAATGCTG	ACTGGAATTCTCAGGAGTCAT TACTTGGGG
Nkrp1f	GTTCCCATCTCCTGTCTACA	AGGAATCAGGACACAGGCTT
Nkrp1g	ATGGATGCACCAGTGCTCTA	TCAGACGTGTTTCAGTGTCT
GAPDH	ACTCACGGCAAATTCAACGGC	ATCACAAACATGGGGGCATCG

were constructed using Advanced Pipmaker (available at http://pipmaker.bx.psu.edu/pipmaker) using single coverage and one strand search settings. Following inputs were used for this analysis: *First sequence mask* file from the output of RepeatMasker; *First sequence exons* file containing the locations of genes, all exons for each *Nkrp1-Clr* gene and gaps within the sequence; *First sequence underlay* file which is similar to *First sequence exons* file with genes, exon numbers and gaps replaced with LightYellow, Green and Red, respectively. A PDF image with the location and orientation for each *Nkrp1-Clr* gene as well as the repetitive elements was generated. The graphical sequence was then assembled into a single figure representing the complete *Nkrp1-Clr* cluster for each of the above three mouse strains using Adobe Illustrator CS4.

2.4 Sequence assembly and alignment

Coding sequences (CDS) for each *Nkrp1*and *Clr* gene were assembled from the BALB/c and 129S6 BAC sequencing file according to the gene annotation. Putative amino acid sequences were translated from each *Nkrp1-Clr* cDNA sequence using the online translation tool ExPASy (available at http://web.expasy.org/translate/). cDNA and amino acid sequence for each *Nkrp1* and *Clr* gene from three different mouse strains, B6, 129S6 and BALB/c, were aligned using the online NCBI blast tool (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5 Southern blot

Restriction fragment length polymorphism (RFLP) analysis was performed on thymic genomic DNA from different mouse strains, which were restriction digested with EcoRI, resolved on 1% agarose gel and transferred to nylon membrane blots. *Clr-b* and *Clr-f* cDNA probes were labeled with P^{32} using NEBlot kit (New England Biolabs, USA). Blots were

probed with P^{32}-labelled *Clr-b* and *Clr-f* cDNA probes in hybridization solution (10% (w/v) dextran sulfate; 1 M NaCl; 1% SDS). Hybridization was carried out overnight at 65 ℃ and post-hybridization washes were performed with 2 × SSC/ 1% SDS solution prewarmed to 65 ℃. The membrane was stripped with 0.2 × SSC/ 0.1% SDS for 30 min at 85 ℃ between different hybridizations.

2.6 Tissue RNA isolation and reverse transcription (RT)-PCR

Adult B6, 129S1 or BALB/c mice were deeply anesthetized with avertin as described previously (129). Mice were then perfused transcardially with PBS until lightened color of the liver was observed. Different tissues of interest (tongue, skeletal muscle, liver, bladder, spleen, thymus, ovary, testis, eye, kidney, heart, lung, lymph node, brain and intestine) were excised, rinsed with PBS, frozen with liquid nitrogen and transferred to -80 ℃ before proceeding to RNA isolation.

RNA was isolated from tissues using RiboZol RNA extraction reagent (AMRESCO) following manufacturer's instructions. 1 μg of RNA was transcribed into cDNA using verso cDNA kit (Thermo Scientific). PCR amplification was performed on 1 μl of cDNA product using primers specific for *Nkrp1-Clr* and *GAPDH* genes (**Table 1**). PCR conditions used were: 94 ℃ 30s, 58 ℃ 30s, 72 ℃ 60s, 35 cycles. PCR products were visualized on 1% agarose gel stained with ethidium bromide. Primer specificity was checked using serial dilution of plasmids (1 ng, 10^{-1} ng, 10^{-2} ng, 10^{-3} ng) containing different *Nkrp1* and *Clr* genes as template for PCR amplification.

2.7 Isolation of intestinal epithelial cells (IECs)

Intestinal epithelial cells (IECs) were isolated from the intestine of adult B6 mice as previously described (130). The jejunum was collected and fat was removed. The intestinal sample was washed with PBS to remove internal content, opened longitudinally, and cut into 0.5 cm pieces. The intestine pieces were then incubated in complete DMEM medium (DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine) with 1 M DTT in 37℃ shaker for 15 min. Cell suspensions were filtered through a 70 μM cell strainer (Fisher Scientific), and filtrate was centrifuged at 300g for 7 min. The cell pellet was resuspended in complete DMEM and kept on ice. The remaining intestine pieces were incubated in PBS with 1.5 M EDTA and shaken for 15 min at 37℃. Cell suspension was filtered as above and filtrate was centrifuged at 300g for 7 min. The cell pellet was resuspended in complete DMEM. Cells from the DTT and EDTA step were combined, layered on discontinuous 20%/40% percoll gradient (GE Healthcare) and centrifuged at 600g for 30 min at 4℃ with minimal acceleration and deceleration. IECs were collected from the interphase of the two percoll gradients. These epithelial cells were collected and transferred to a new tube filled with PBS and then spun at 500g for 5 min. The cell pellet was then resuspended in PBS and used for flow cytometry analysis.

2.8 Flow cytometry and cell sorting

3×10^7 intestinal epithelial cells were spun down at 500g for 5 min, resuspended in FACS buffer (PBS, 0.5% BSA and 0.02% NaN_3), transferred to FACS tubes and spun down at 500g for 5 min. The cell pellet was resuspended in 8 μl mouse BD Fc block (BD Pharmingen) by gentle vortexing followed by 15 min incubation at 4℃. Anti-LFA-1 antibody conjugated to FITC fluorochrome was added to the cells which were then incubated in the dark for 20 min at 4℃. After antibody staining, the cells were washed with FACS

buffer and spun down at 500g for 5 min. The cell pellet was then resuspended in FACS buffer at a final cell concentration of 1×10^7 cells/ml. Cells were kept in the dark on ice until they were sorted into LFA-1^+ lymphocyte and LFA-1^- IEC fractions using a MoFlow cell sorter (Beckman Coulter, USA) at the Ottawa Hospitals Research Institute Flow Cytometry Facility.

2.9 Semi-quantitative RT-PCR

RNA was isolated from unsorted IECs, sorted LFA-1^- IECs and LFA-1^+ lymphocytes and 1 μg of RNA was transcribed into cDNA as described above. Semi-quantitative PT-PCR was performed on serial four-fold dilutions of the stock cDNA using primers specific for *Clr-f* and *GAPDH* (**Table 1**).

2.10 *In situ* hybridization

In situ hybridization analysis for *Clr* genes in different tissues were performed as described previously (131). Plasmids containing *Clr* genes were either generated by cloning or were isolated from the clones previously made in our lab. In order to generate antisense and sense RNA probes, plasmids were sequenced to identify ones with *Clr* genes inserted in both orientations. Digoxigenin-labeled antisense and sense RNA probes were transcribed from linearized plasmids containing *Clr* genes with T7 polymerases using DIG RNA labeling mix (Roche). RNA probes were purified with commercially available RNA purification columns (RNeasy Mini Kit, Qiagen). Purified probes were tested for integrity on a standard 1% agarose/ethidium bromide gel, quantitated using a Nanodrop DNA/RNA quantification system (Thermo Scientific) and aliquots were stored at -80 ℃.

Tissues were collected from adult B6 mice and fixed either in 10% formalin at room temperature or in 4% paraformaldehyde at 4 ℃ with gentle shaking for up to 24 h. Tissues

were then sent to the Histology Core Facility (Roger Guindon Hall, University of Ottawa, Ontario) for paraffin embedding and 4 μm sections were prepared on glass slides.

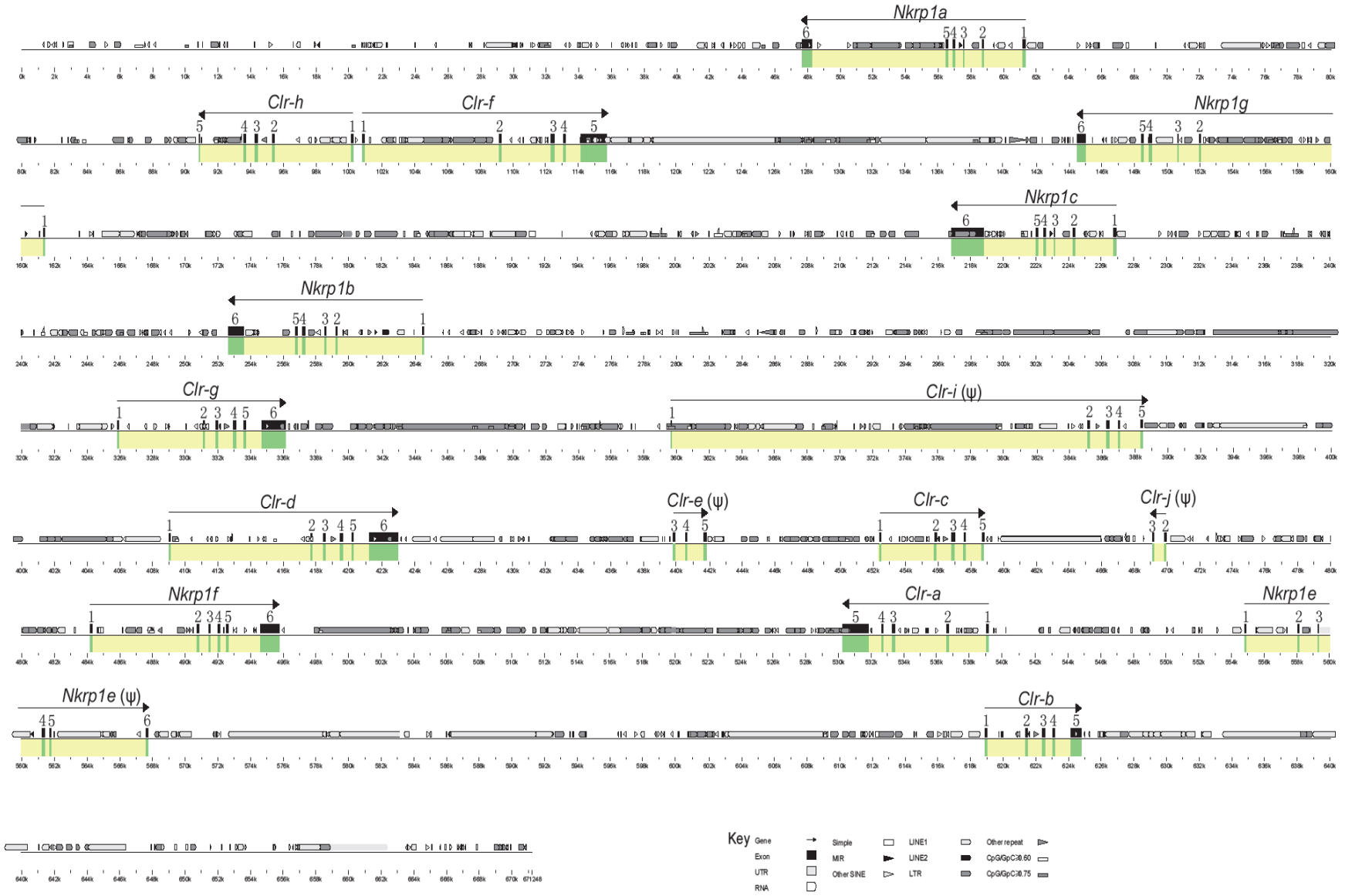
Tissue slides were deparaffinated, treated with 0.2 N HCl for 15 min and 30 μg/ml proteinase K at 37 ℃ for 20 min, postfixed in 4% paraformaldehyde for 10 min and incubated with 0.25% acetic anhydride in 0.1 M triethanolamine solution twice for 5 min each. Slides were then pre-hybridized with hybridization solution (50% (v/v) formamide/5× SSC pH 4.5; 2% (w/v) blocking powder (Roche); 0.05% (w/v) CHAPS; 5 mM EDTA; 50 μg/ml heparin; 1 μg/ml yeast RNA) in a 58 ℃ oven for at least 1 h, and then incubated with hybridization solution containing 500 ng/ml digoxigenin-labeled probe overnight at 58 ℃. Post-hybridization washes were performed with 2 × SSC pH 7.5 followed by washing with 50% formamide/2 × SSC pH 4.5 at 55 ℃ three times for 20 min each. Slides were stained with sheep anti-digoxigenin alkaline phosphatase-conjugated antibody at 1/1000 dilution in blocking solution (Roche) at 4 ℃ overnight. After several washes in Tris/NaCl buffer, slides were equilibrated in NTM buffer (0.1 M Tris-Cl pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$). Color development was performed by adding nitro blue tetrazolium /5-bromo, 4-chloro, 3-indoylphosphate (NBT/BCIP, Roche) substrates to the slides and slides were kept in the dark at room temperature until optimal staining. Slides were then counterstained with lightgreen or methylgreen and visualized using a light microscope.

3 Results

3.1 Genomic organization of the B6 *Nkrp1-Clr* gene cluster

Previous studies have shown that the related *Ly49* genes are highly polymorphic in terms of gene content and gene order among different mouse strains including B6, 129, BALB/c and NOD (89-92). To study the genomic conservation of the *Nkrp1-Clr* gene cluster, the B6 *Nkrp1-Clr* cluster was assembled from B6 genomic sequence available at UCSC genome browser database. A representation of the gene content and gene organization of the B6 *Nkrp1-Clr* cluster is shown in **Figure 3**. The B6 *Nkrp1-Clr* cluster covers a range of approximately 578kb, with the following gene order: *Nkrp1a*, *Clr-h*, *Clr-f*, *Nkrp1g*, *Nkrp1c*, *Nkrp1b*, *Clr-g*, *Clr-i*, *Clr-d*, *Clr-e*, *Clr-c*, *Clr-j*, *Nkrp1f*, *Clr-a*, *Nkrp1e* and *Clr-b*. The *Nkrp1* gene family members have six exons while most of the functional *Clr* family members have five exons with the exception of *Clr-g* and *Clr-d* which have six exons. *Nkrp1e* and *Clr-i* are pseudogenes due to the presence of many early stop codons in their open reading frame. *Clr-e* and *Clr-j* are also pseudogenes due to only having partial exons. It remains unknown whether *Clr-h* is a functional gene, as no transcript data is available. Nevertheless, the analysis of the coding sequence of the *Clr-h* gene indicates that it has the potential to be translated into a functional protein as no early stop codons are present within its open reading frame. Interestingly, *Clr-h* and *Clr-f* genes are closely located in the genome but with opposite transcript orientation, suggesting the possibility that the promoter sequences for *Clr-h* and *Clr-f* genes may reside within the *Clr-f* and *Clr-h* genes, respectively.

Figure 3: Genomic organization of the B6 *Nkrp1-Clr* cluster. Advanced Pipmaker was used to construct a scale diagram of the exons and a PIP against itself. Genes and exons are marked in lightyellow and green, respectively. The length of the sequence in kilobases is shown underneath the PIP. Arrows show gene orientation, with gene name above the arrows. Obvious pseudogenes are denoted with a Ψ. The locations of various kinds of repetitive elements were revealed with RepeatMasker and are shown (see key).


Nkrp1a
Clr-h
Clr-f
Nkrp1g
Nkrp1c
Nkrp1b
Clr-g
Clr-i (ψ)
Clr-d
Clr-e (ψ)
Clr-c
Clr-j (ψ)
Nkrp1f
Clr-a
Nkrp1e
Nkrp1e (ψ)
Clr-b
Key
Gene
Exon
UTR
RNA
Simple
MIR
Other SINE
LINE1
LINE2
LTR
Other repeat
CpG/GpC≥0.60
CpG/GpC≥0.75

3.2 Repeats in *Nkrp1-Clr* and *Ly49* gene clusters from B6 mice

It was previously proposed that the high polymorphism of the *Ly49* gene cluster is due to the high percentage of repeat elements (50%) within this region compared to levels in the whole mouse genome (39%) (88-92, 132). Notably, the most abundant LINE1 element (36%) present within the *Ly49* cluster is greater than that within the whole mouse genome (19%) (132). This high rate of LINE1 insertion within the *Ly49* gene cluster may lead to unequal homologous recombination events which may drive gene duplication and the evolution of the Ly49 receptor repertoire (92). Thus, we decided to study the repeat elements within the *Nkrp1-Clr* gene cluster and compare them with the related *Ly49* gene cluster. The different types of repeat elements within the B6 *Nkrp1-Clr* and *Ly49* gene cluster are summarized in **Table 2**. The overall percentage of repeat elements within the *Nkrp1-Clr* cluster is 54%, which is similar to that within the *Ly49* cluster (50%). There is no difference in the percentage of some repeat elements such as SINEs, small RNA, simple repeats, etc. Interestingly, the percentage of the more abundant LINE1 element and LTR subfamily ERV_classII are different between the *Nkrp1-Clr* and the *Ly49* gene cluster. Specifically, the LINE1 element within the *Nkrp1-Clr* gene cluster is 22% which is comparable to the 19% of LINE1 within the whole mouse genome and is much lower than that within the *Ly49* gene cluster (36%). This is in agreement with the hypothesis that LINE1 may play a role in driving the evolution of the Ly49 receptors and that the *Nkrp1-Clr* gene family may not be as polymorphic as the related *Ly49* gene cluster. Notably, the ERV_classII within the *Nkrp1-Clr* gene cluster is more than twice the percentage of that within the *Ly49* gene cluster, of which the significance remains unknown.

Table 2: Summary of repeats in *Nkrp1-Clr* and *Ly49* gene clusters from B6 mice[a]

Type of element	Number of elements		Length occupied (bp)		Percentage of sequence (%)	
	Nkrp1-Clr[b]	*Ly49*[c]	*Nkrp1-Clr*	*Ly49*	*Nkrp1-Clr*	*Ly49*
SINES						
Alu/B1	94	99	11303 bp	11148 bp	1.68 %	1.95 %
B2-B4	106	35	15205 bp	4936 bp	2.27 %	0.86 %
IDs	12	5	832 bp	376 bp	0.12 %	0.07 %
MIRs	9	0	1207 bp	0 bp	0.18 %	0.00 %
Total	221	139	28547 bp	16460 bp	4.25 %	2.87 %
LINEs						
LINE1	148	264	147150 bp	203094 bp	21.92 %	35.46 %
LINE2	9	4	1133 bp	348 bp	0.17 %	0.06 %
L3/CR1	1	0	46 bp	0 bp	0.01 %	0.00 %
Total	158	268	148329 bp	203442 bp	22.10 %	35.52 %
LTR elements						
ERVL	21	2	5500 bp	791 bp	0.82 %	0.14 %
ERVL-MaLRs	57	26	17394 bp	12857 bp	2.59 %	2.24 %
ERV_classI	38	18	18508 bp	14752 bp	2.76 %	2.58 %
ERV_classII	182	43	136954 bp	40436 bp	20.40 %	7.06 %
Total	298	89	178356 bp	68836 bp	26.57 %	12.02 %
DNA elements						
hAT-Charlie	6	4	902 bp	305 bp	0.13 %	0.05 %
TcMar-Tigger	17	0	3784 bp	0 bp	0.56 %	0.00 %
Total	25	4	4939 bp	305bp	0.74 %	0.05 %
Unclassified	2	1	1866 bp	45 bp	0.28 %	0.01 %
Total interspersed repeats			362037 bp	289088 bp	53.93 %	50.48 %
Small RNA	14	8	1028 bp	513 bp	0.15 %	0.09 %
Satellites	3	2	313 bp	197 bp	0.05 %	0.03 %
Simple repeats	186	199	10491 bp	12840 bp	1.56 %	2.24 %
Low complexity	64	110	3236 bp	5281 bp	0.48 %	0.92 %

a. As detected by Repeatmasker.
b. The region scanned for repeats in the *Nkrp1-Clr* cluster was 671 kb and included all genes between exon 2 of *Ovos* and exon 5 of *Clr-b*.
c. The region analyzed for the *Ly49* cluster was from the beginning of the *α* gene fragment to exon 6 of *Ly49q* (572 kb).

3.3 *Clr* gene conservation

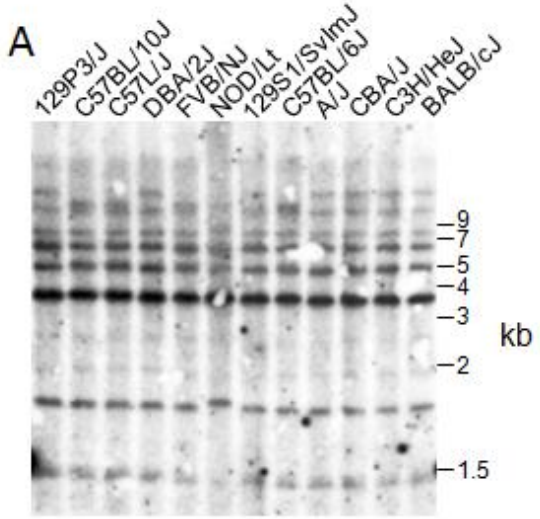
Previous aCGH and RFLP analyses of the *Nkrp1* genes in different inbred mouse strains reveal that the *Nkrp1* genes fall into four subgroups (133, 134). To study the RFLP pattern and the conservation of *Clr* genes, we performed RFLP analysis of genomic DNA from 12 different inbred mouse strains using *Clr-b* and *Clr-f* probes. Southern blot shows that *Clr-b* and *Clr-f* are highly conserved in different mouse strains studied despite the presence of some polymorphisms such as in the NOD mice (**Figure 4**). Therefore, all members of the *Clr* gene family seem to be conserved due to cross hybridization of *Clr-b* and *Clr-f* probes with other closely related *Clr* genes.

3.4 Analysis of the 129S6 and BALB/c *Nkrp1-Clr* gene cluster

In previous studies, physical maps of the *Nkrp1-Clr* gene cluster in 129S6 and BALB/c mouse strains were constructed using BAC clones containing *Nkrp1* and *Clr* genes which are generated from 129S6 and BALB/c-derived genomic library, respectively (109, 122). The overall gene content and gene organization of the *Nkrp1-Clr* gene cluster in the above two mouse strains were similar to that in B6 mice. However, some genes which are actually present in 129S6 or BALB/c mice may be missing in gene mapping studies. Therefore, in order to gain further understanding of the genomic conservation of the *Nkrp1-Clr* gene cluster, the complete *Nkrp1-Clr* gene cluster from 129S6 and BALB/c mice was determined using sequencing. Specifically, a minimal number of overlapping BAC clones, which cover the whole *Nkrp1-Clr* region, were chosen from the 129S6 and BALB/c gene maps for shotgun sequencing. A representation of the gene content and gene organization for the *Nkrp1* and *Clr* genes in 129S6 and BALB/c mice is shown in **Figure 5A and 5B**, respectively. The 129S6 and BALB/c *Nkrp1-Clr* gene clusters both include all genes present in the B6 *Nkrp1-Clr* gene cluster (**Figure 3**). In addition, the *Nkrp1-Clr* gene cluster has the

same gene order and gene orientation in the three mouse strains. PIP analysis of the 129S6 and BALB/c *Nkrp1-Clr* cluster against B6 showed that *Nkrp1-Clr* haplotypes are highly similar in these three mouse strains.

Figure 4: Southern blot analysis of the conservation of *Clr* genes in mice. Thymus genomic DNA from different inbred mouse strains digested with EcoRI was blot using P^{32} labeled (A) *Clr-b* and (B) *Clr-f* probe, respectively.


A
129P3/J
C57BL/10J
C57L/J
DBA/2J
FVB/NJ
NOD/LtJ
129S1/SvImJ
C57BL/6J
A/J
CBA/J
C3H/HeJ
BALB/cJ
9
7
5
4
3
2
1.5
kb

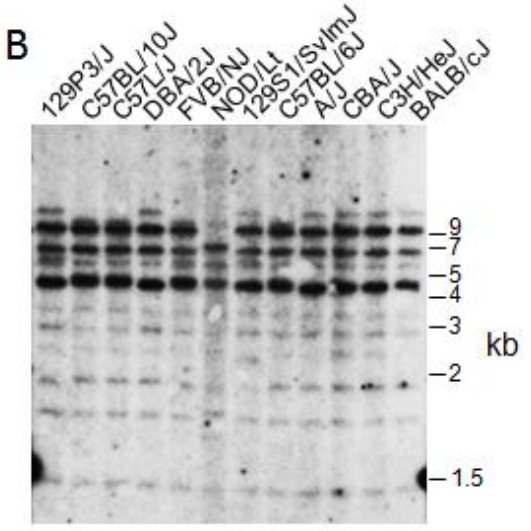
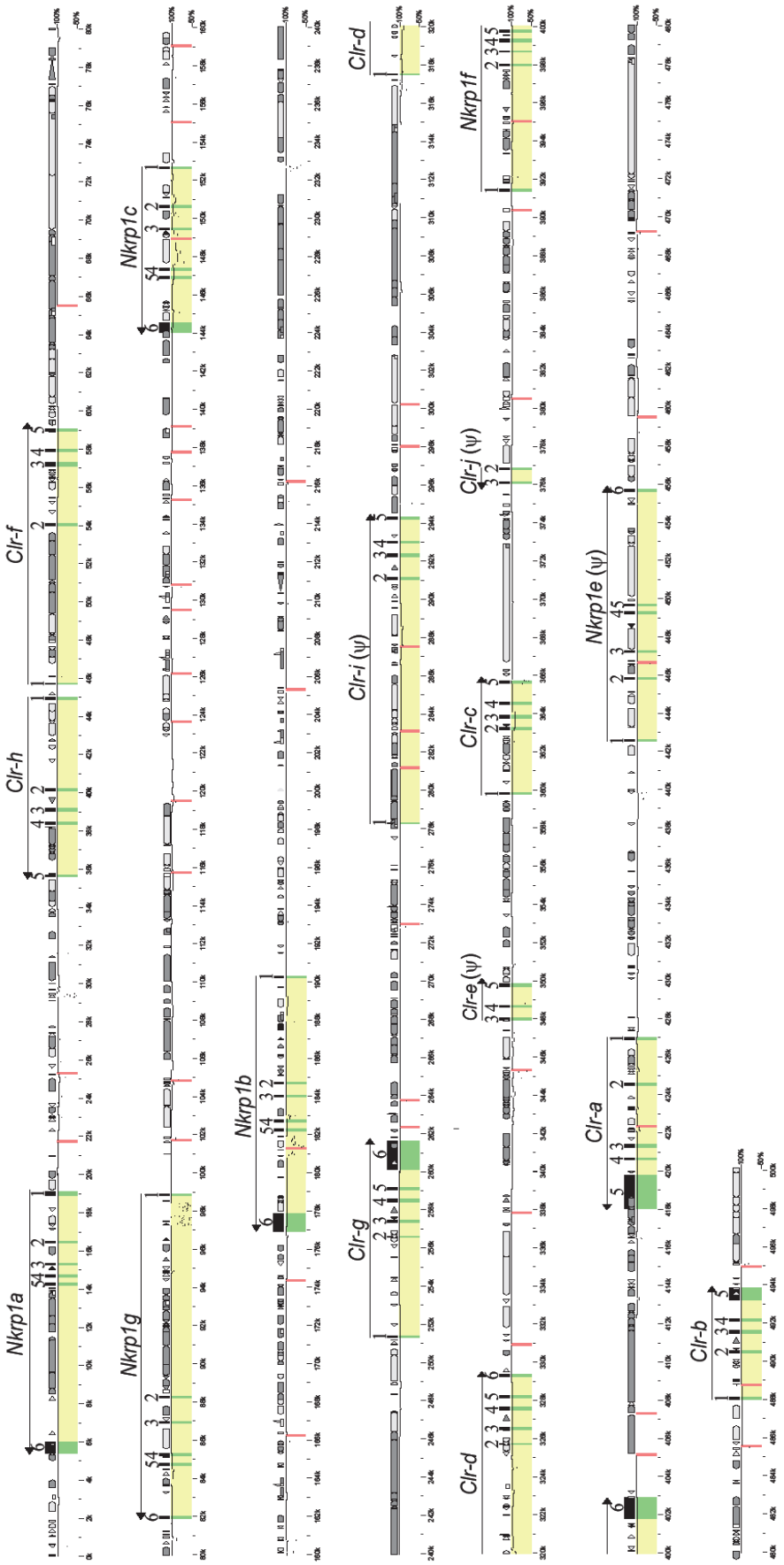
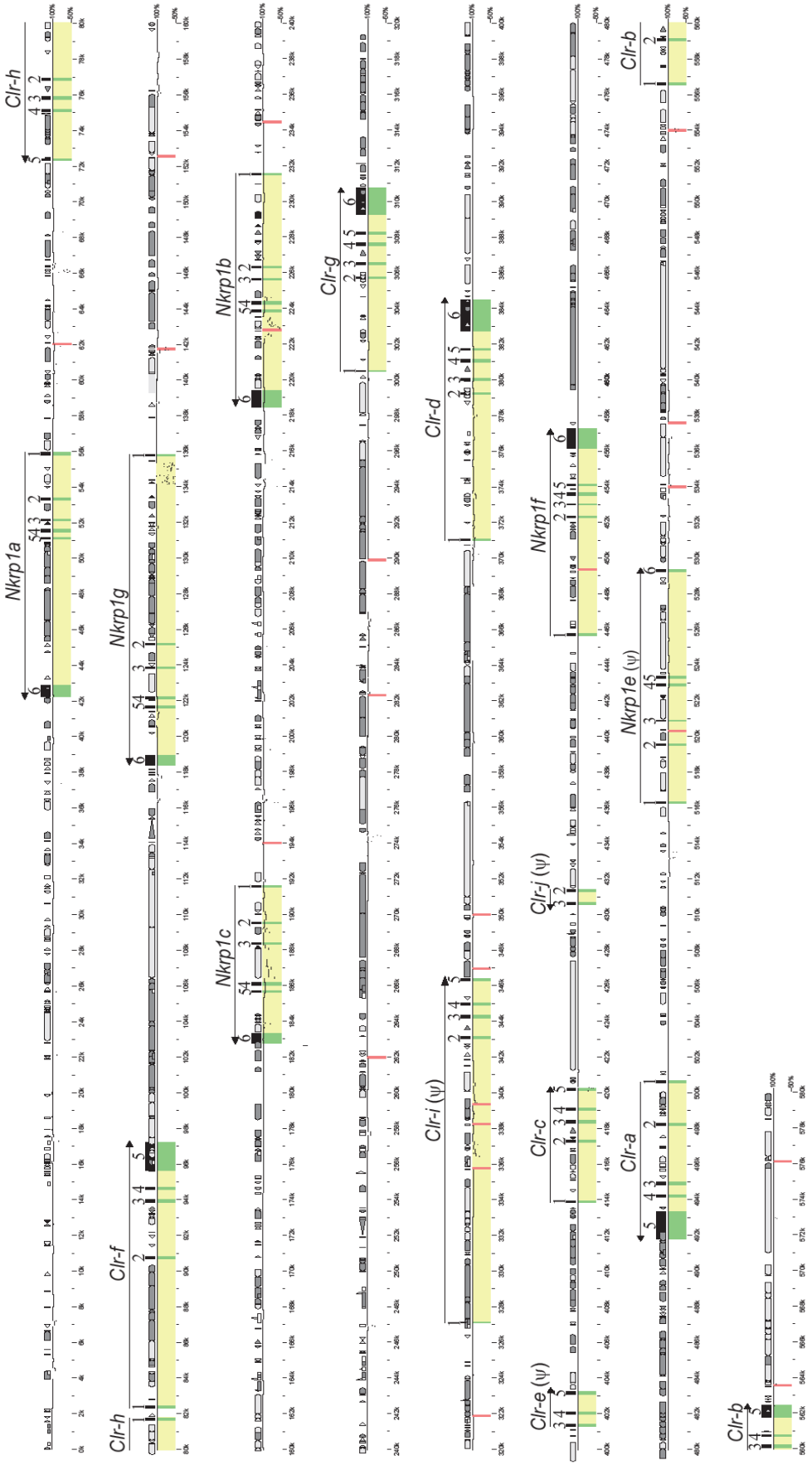

B
129P3/J
C57BL/10J
C57L/J
DBA/2J
FVB/NJ
NOD/LtJ
129S1/SvImJ
C57BL/6J
A/J
CBA/J
C3H/HeJ
BALB/cJ
9
7
5
4
3
2
1.5
kb

Figure 5: Genomic organization of the 129S6 (A) and BALB/c (B) *Nkrp1-Clr* cluster. Advanced Pipmaker was used to construct a scale diagram of the exons and a PIP against the B6 *Nkrp1-Clr* gene cluster sequence. Genes and exons are marked in lightyellow and green, respectively. Red vertical lines indicate gaps in the assembly. The length of the sequence in kilobases is shown underneath the PIP. Arrows show gene orientation, and the gene name is above the arrow. Obvious pseudogenes are denoted with a Ψ. The locations of various kinds of repetitive elements were revealed with RepeatMasker and are shown (Refer to Figure 3 for keys).

A


Nkrp1a
Nkrp1g
Clr-h
Clr-f
Nkrp1c
Nkrp1b
Clr-g
Clr-d
Clr-i (ψ)
Clr-e (ψ)
Clr-d
Clr-c
Clr-j (ψ)
Nkrp1f
Nkrp1e (ψ)
Clr-a
Clr-b

B


Nkrp1a
Clr-h
Nkrp1g
Clr-f
Clr-h
Nkrp1c
Nkrp1b
Clr-g
Clr-d
Clr-i (ψ)
Clr-c
Clr-j (ψ)
Nkrp1f
Clr-e (ψ)
Clr-a
Nkrp1e (ψ)
Clr-b
Clr-b

3.5 *Nkrp1* and *Clr* gene allelic polymorphism

In order to study the allelic polymorphism for each *Nkrp1* and *Clr* gene, coding sequences were assembled from the sequenced *Nkrp1-Clr* gene cluster of 129S6 and BALB/c mice. Putative amino acid sequences were translated from the assembled coding sequences. The comparison of all potential functional *Nkrp1-Clr* genes among three different mouse strains is shown in **Table 3**.

Most of the *Nkrp1* and *Clr* genes have more than 98% identity among three mouse strains studied with focused diversity in *Clr-c*, *Nkrp1b/d* and *Nkrp1c*. In addition, most of the genes are 100% identical between 129S6 and BALB/c mouse strains and differ from the B6 counterparts with the exception of *Clr-c* and *Nkrp1f*, which are 100% identical between BALB/c and B6, and differ from their 129S6 counterparts. Finally, *Clr-a* is different among three mouse strains. Therefore, the BALB/c and 129S6 *Nkrp1-Clr* genes seem to be more similar and are distinct from their B6 counterparts except for *Clr-c*, *Nkrp1f* and *Clr-a*. Interestingly, *Clr-h* is 100% conserved in three mouse strains, indicating that Clr-h may be translated into a protein that is functionally conserved. However, no *Clr-h* transcript has been detected in any tissue yet.

Amino acid comparison shows that Clr-c is highly polymorphic in 129S6 mice compared to B6 and BALB/c mice, with a 38 amino acid difference. NKR-P1B/D and NKR-P1C are conserved in 129S6 and BALB/c mice, and have a 23 and 26 amino acid difference compared to B6 mice, respectively.

In summary, compared to *Ly49* genes, the *Nkrp1* and *Clr* genes are highly conserved with focused diversity, indicating that these relatively polymorphic genes may undergo evolutionary selection.

Table 3: *Nkrp1-Clr* gene allelic polymorphism. cDNA sequences for *Nkrp1* and *Clr* genes from 129S6, B6 and BALB/c mouse strains were assembled and putative amino acid sequences were translated. Pair-wise alignment of the cDNA and amino acid sequence for each *Nkrp1* and *Clr* gene was performed using BlastN. The percent identity for cDNA and amino acid sequence of each gene is shown.

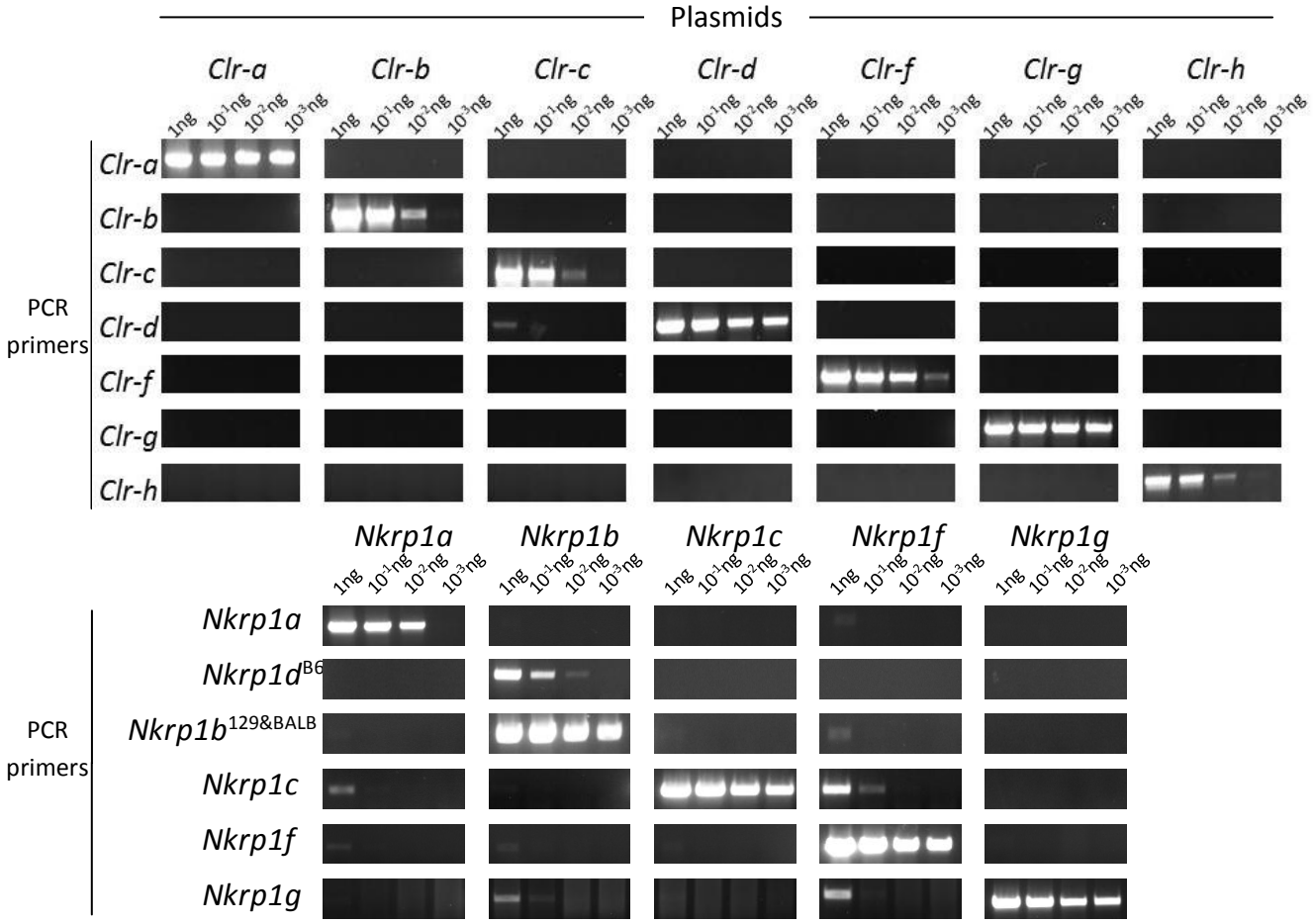
	cDNA			Amino acid		
	129S6 vs B6	BALB/c vs B6	129S6 vs BALB/c	129S6 vs B6	BALB/c vs B6	129S6 vs BALB/c
Clr-a	98%	99%	99%	97%	99%	97%
Clr-b	100%	100%	100%	100%	100%	100%
Clr-c	92%	100%	92%	82%	100%	82%
Clr-d	98%	98%	100%	97%	97%	100%
Clr-f	99%	99%	100%	99%	99%	100%
Clr-g	100%	100%	100%	100%	100%	100%
Clr-h	100%	100%	100%	100%	100%	100%
Nkrp1a	99%	99%	100%	99%	99%	100%
Nkrp1b(d)	96%	96%	100%	90%	90%	100%
Nkrp1c	94%	94%	100%	88%	88%	100%
Nkrp1f	99%	100%	99%	99%	100%	99%
Nkrp1g	99%	99%	100%	99%	99%	100%

3.6 *Nkrp1* and *Clr* transcript expression in different tissues

Previous microarray studies identified the tissue or cell specific expression for several *Nkrp1* and *Clr* genes (data available at http://www.biogps.org/) (135). However, only limited transcript expression studies for the *Nkrp1-Clr* genes are available apart from this. As the above genomic analysis has revealed the complete gene content and sequence of individual *Nkrp1* and *Clr* genes for three different mouse strains, further work was done to determine and expand the transcript expression profile for all potentially functional *Nkrp1* and *Clr* genes, and to confirm the available microarray data. Specifically, 14 different tissues were chosen for RT-PCR analysis, namely: tongue, skeletal muscle, liver, bladder, spleen, thymus, ovary/testis, eye, kidney, heart, lung, lymph node, brain and intestine from three different mouse strains, B6, 129S1 and BALB/c.

The specificity of the primers used in amplifying each *Nkrp1* and *Clr* gene was tested against serially diluted *Nkrp1-Clr* cDNA containing plasmid (**Figure 6**). For primers amplifying the *Clr* genes, only the *Clr-d* primer had weak cross-reactivity with *Clr-c* containing plasmid; for primers amplifying the *Nkrp1* genes, the following primer:plasmid cross reactivity was detected: *Nkrp1b*$^{129\&BALB/c}$:*Nkrp1f*, *Nkrp1c*:*Nkrp1a/f*, *Nkrp1f*:*Nkrp1a/b*, *Nkrp1g*:*Nkrp1b/f*. However, cross reactions were only detected with the highest amount of template plasmid (1ng) or in some cases weak cross reactions were observed with 0.1ng of template plasmid. When amplifying *Nkrp1* and *Clr* genes from tissues, only about 50ng of tissue cDNA will be used, and which also contains many copies of numerous other genes; thus, the cross reaction detected with plasmids as PCR template will not be observed when performing gene transcript profiling in tissues.

Figure 6: PCR analysis of *Nkrp1-Clr* primer specificity. RT-PCR was performed on cloned plasmid templates which were serially diluted (1ng, 10^{-1}ng, 10^{-2} ng, 10^{-3} ng) and contained different *Nkrp1* and *Clr* genes, with primers for each of the genes. PCR products were run on the standard 1% agarose/ethidium bromide gel.


Plasmids
Clr-a
Clr-b
Clr-c
Clr-d
Clr-f
Clr-g
Clr-h
1ng
10-1ng
10-2ng
10-3ng
PCR primers
Nkrp1a
Nkrp1b
Nkrp1c
Nkrp1f
Nkrp1g
Nkrp1d B6
Nkrp1b 129&BALB

Results of expression analysis for *Nkrp1* and *Clr* transcripts are shown in **Table 4** with the microarray data from Biogps underneath the RT-PCR results. No microarray data is available for *Clr-c*, *Clr-h* and *Nkrp1g* (136). Overall, the expression of *Nkrp1* and *Clr* genes in three different mouse strains are consistent and agree well with the microarray data (**Table 4**).

Clr-a transcript is only detected in the intestine and not in other tissues. *Clr-b* is broadly expressed in every tissue except for the brain. *Clr-c* has low expression in the tongue, spleen, thymus, ovary/testis, lymph node and intestine. *Clr-d* is specifically expressed in the eye. *Clr-g* is expressed in the spleen, thymus, lung, lymph node and intestine. However, the microarray data is negative for *Clr-g* expression in the intestine and lung. *Clr-h* transcript is not detected in any of the tissues for all three mouse strains studied despite its intact open reading frame.

For *Nkrp1s*, their transcripts are detected in the intestine, spleen, thymus, lung and lymph node with the exception that *Nkrp1a* is not expressed in the intestine and *Nkrp1g* is not detected in the lung and lymph node. Additionally, *Nkrp1c*, *Nkrp1b/d* and *Nkrp1f* are detected in the liver. *Nkrp1c* is found in the ovary/testis. *Nkrp1b/d* is more broadly expressed in the tongue, bladder, ovary/testis, and heart. However, the microarray data is not consistent with RT-PCR in that *Nkrp1a* was not detected in the thymus, ovary and lung; *Nkrp1c* was not detected in the intestine; *Nkrp1f* was not detected in the liver, thymus and lung.

Table 4: RT-PCR analysis of *Clr* (A) and *Nkrp1* (B) transcript expression in different tissues of B6, 129S1 and BALB/c mouse strains. PCR products were amplified from cDNA prepared from equal amount of RNA (1 µg).

A)

	tongue	muscle	liver	bladder	spleen	thymus	ovary/testis	eye	kidney	heart	lung	lymph node	brain	intestine
Clr-a														
B6	-	-	-	-	-	-	-	-	-	-	-	-	-	+
BALB	-	-	-	-	-	-	-	-	-	-	-	-	-	+
microarray[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Clr-b														
B6	+	+++	++	+	++	++	+	+	+	+	+	++	-	+
129	+	+++	+++	+	+	+	+	-/+	++	++	++	++	-	+
BALB	+	+++	+++	+	++	++	+	-/+	++	+++	++	++	-	+
microarray	+	+	+	+	+	+	+	-	+	+	+	+	-	+
Clr-c														
B6	-/+	-	-	-/+	+	+	+	-	-	-	-	-/+	-	+
129	-/+	-	-	-/+	+	+	-/+	-	-/+	-	-	+	-	+
BALB	+	-	-	-	+	+	+	-	-/+	-	+	-/+	-	+
microarray								ND[b]						
Clr-d														
B6	+	-	-	-	-/+	-/+	-	+	-	-	-	-	-	-
129	-	-	-	-	-/+	-	-	+	-	-	-	-	-	-
BALB	-/+	-	-/+	-/+	-/+	-/+	-	+	-	-	-	-	-	-
microarray	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Clr-f														
B6	-/+	-/+	++	-	-/+	-/+	-	-	+++	-	-/+	-/+	-	+++
129	-	-	++	-/+	+	-	-	-	+++	+	-	-/+	-/+	+++
BALB	-	-/+	-	-	-	-	-	-	+++	-	-	-/+	-	+++
microarray	-	+	+	-	-	-	-	-	+	-	-	-	-	+
Clr-g														
B6	-	-	-	-	++	++	-	-	-/+	-	+	++	-	+
129	-	-	-/+	-	++	+++	-/+	-	-/+	-	+	+++	-	+
BALB	-	-/+	-/+	-	++	++	-	-	-	-	+	++	-/+	++
microarray	-	-	-	-	+	+	-	-	-	-	-	+	-	-
Clr-h														
B6	-	-	-	-	-	-	-	-	-	-	-	-	-	-
129	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BALB	-	-	-	-	-	-	-	-	-	-	-	-	-	-
microarray								ND						

B)

	tongue	muscle	liver	bladder	spleen	thymus	ovary/testis	eye	kidney	heart	lung	lymph node	brain	intestine
Nkrp1a														
B6	-	-	-	-	+	+	+	-	-	-	+	++	-	-
129	-	-	-	-	+	+	++	-	-	-	+	++	-	-
BALB	-	-	-/+	-	-/+	-/+	+	-	-	-	++	+	-	-
microarray	-	-	-	-	+	-	-	-	-	-	-	+	-	-
Nkrp1c														
B6	-	-	+	-/+	+++	++	-/+	-	-/+	-	++	++	-	+
129	-	-	+	-/+	++	++	-/+	-/+	-/+	-/+	+	++	-	+
BALB	-	-	++	-/+	+	+	+	-	-/+	+	++	+	-	+
microarray	+	-	+	-	+	+	-	-	-	-	+	+	-	-
Nkrp1d/b														
B6	+	-/+	+	+	++	++	+	-/+	+	+	++	++	-	++
129	+	-	-	-/+	+	+	+	-/+	-	+	++	++	-	+
BALB	+	-	+	+	+	+	-/+	-/+	-	+	++	+	-	+
microarray	+	+	+	+	+	+	-	+	+	+	+	+	-	+
Nkrp1f														
B6	-	-	+	-	++	+	-	-	-	-	++	++	-	+
129	-	-	+	-	+	+	-	-	-	-	+	+++	-	+
BALB	-	-	++	-	+	+	-	-	-	-	++	++	-	+
microarray	-	-	-	-	+	-	-	-	-	-	-	+	-	+
Nkrp1g														
B6	-	-	-	-	+	+	-	-	-	-	+	-/+	-	+
129	-	-	-	-	+	+	+	-	-	-	-	-/+	-/+	+
BALB	-	-	-	-	+	+	+	-	-	-	-	-	-	+
microarray								ND						

-, no expression; -/+, no expression or very weak expression; +, weak to moderate expression; ++, strong expression; +++, very strong expression.

a. Microarray data were obtained from the Mouse MOE430 Gene Atlas database at Biogps portal server in order to compare with RT-PCR results.
b. ND, not determined.

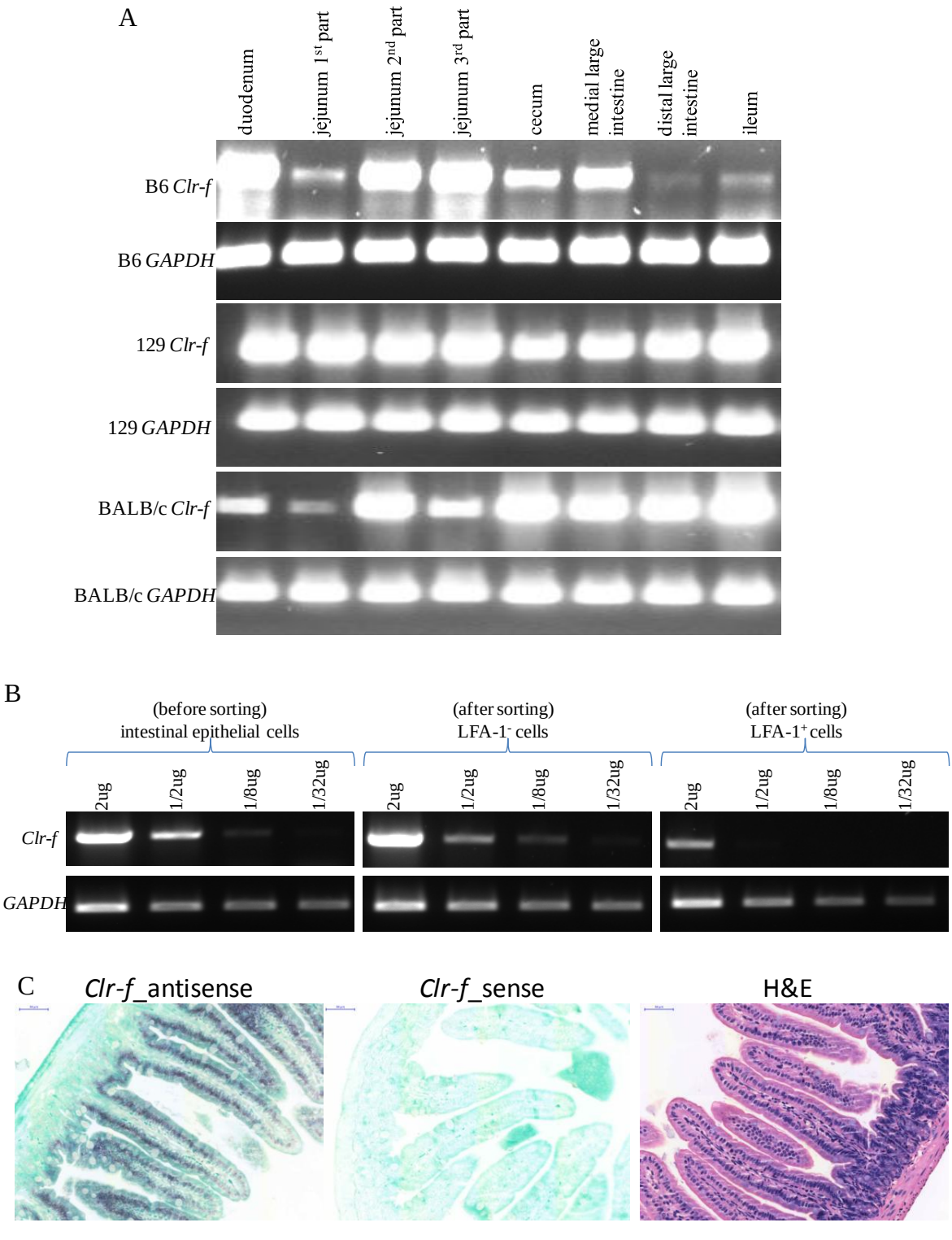
3.7 Cell type specific expression of *Clr-f*

To our surprise, a very high expression of *Clr-f* was detected in the intestine with RT-PCR. Further work was performed to test for expression of *Clr-f* in different parts of the intestine and to identify specific cell types where *Clr-f* is expressed. Firstly, RT-PCR was performed on cDNA prepared from different parts of the intestine including the duodenum, jejunum, ileum, cecum, medial large intestine and distal large intestine from three different inbred mouse strains (**Figure 7A**). The results show that *Clr-f* is highly and consistently expressed in all parts of the intestine from all three mouse strains. We next determined the nature of cells in the intestine expressing *Clr-f*. Cell preparation from the jejunum of B6 mice was labeled with LFA-1 mAb and sorted into LFA-1^+ lymphocytes and LFA-1^- cells (mainly IECs). RT-PCR was then performed on cDNA prepared from the sorted cells. *Clr-f* was found to be highly expressed in the non-lymphocyte populations which are mainly composed of IECs (**Figure 7B**). Finally, to confirm *Clr-f* expression in the non-lymphocyte population, *in situ* hybridization was carried out on paraffin-embedded intestinal sections of B6 mice using a DIG-labeled *Clr-f* specific antisense probe. **Figure 7C** shows the *in situ* hybridization profile for *Clr-f* transcripts in the intestine. High expression of *Clr-f* transcripts was detected in the epithelial cells of the intestinal villi.

3.8 *In situ* hybridization for *Clr* genes in various tissues

Since *Clr-f* was found to be highly expressed in intestinal epithelial cells, which are non-hematopoietic cells, it prompted us to study cell-specific expression of *Clr* genes. Due to the lack of antibodies for most of the Clr proteins except for Clr-b (110), *in situ* hybridization was used to detect *Clr* transcripts in specific cell types in various tissues.

Figure 7: *Clr-f* is expressed in the intestinal epithelial cells. (A) Different parts of the whole intestine were collected from B6, 129S1 and BALB/c mice for RNA isolation; RT-PCR was performed using *Clr-f* and *GAPDH* specific primers. (B) *Clr-f* transcript is abundant in non-lymphocytes (mainly IECs). Intestinal epithelial cells were isolated from B6 mice followed by staining with LFA-1 mAb. Cells were sorted into lymphocyte and non-lymphocyte populations. RNA was isolated from the above sorted and unsorted cells and RT-PCR was performed to analyze the expression of *Clr-f*. (C) *Clr-f* is expressed in the intestinal epithelial cells. *In situ* hybridization was performed to detect the *Clr-f* gene in the intestine. 4µm thick intestine paraffin-embedded sections were permeabilized and hybridized with 500ng/ml of *Clr-f* antisense and sense (negative control) probes labeled with digoxigenin. Color development was performed using anti-DIG-AP conjugated antibody followed by adding NBT/BCIP substrate, which forms a purple precipitate on the slides. H&E staining for the small intestine is shown to visualize the tissue structure. The scale bar indicates 50 µm.


A
duodenum
jejunum 1st part
jejunum 2nd part
jejunum 3rd part
cecum
medial large intestine
distal large intestine
ileum
B6 Clr-f
B6 GAPDH
129 Clr-f
129 GAPDH
BALB/c Clr-f
BALB/c GAPDH
B
(before sorting) intestinal epithelial cells
(after sorting) LFA-1- cells
(after sorting) LFA-1+ cells
2ug
1/2ug
1/8ug
1/32ug
Clr-f
GAPDH
C
Clr-f_antisense
Clr-f_sense
H&E

In addition to IECs (**Figure 7C**) *Clr-f* is expressed in the tubular epithelial cells of the kidney (**Figure 8**).

Clr-b transcripts were found to be broadly expressed in different tissues (**Figure 9**). It is highly expressed in lymph node, the white pulp area of the spleen, thymus and the hepatocytes close to the epithelial layer of the liver and those around the central vein and portal triad area. In addition, *Clr-b* transcripts are detected in the glomeruli of the kidney, lung epithelial cells and myocytes of the skeletal muscle and heart. *Clr-g* which is closely related to *Clr-b* is not detected in the skeletal muscle by RT-PCR, thus, a *Clr-g* probe was used as negative control in *in situ* hybridization on skeletal muscle to test probe specificity. As expected, no detectable staining was observed in the skeletal muscle with the *Clr-g* probe (**Figure 9**). Our study for *Clr-b* expression is in accordance with a previous report showing expression of *Clr-b* in the lung, heart, skeletal muscle, and spleen (125). However, they did not detect *Clr-b* in the kidney or liver, which disagrees with our results. In addition, the brain shows positive staining of *Clr-b* in their study while our RT-PCR result is negative for *Clr-b* expression in the brain (125).

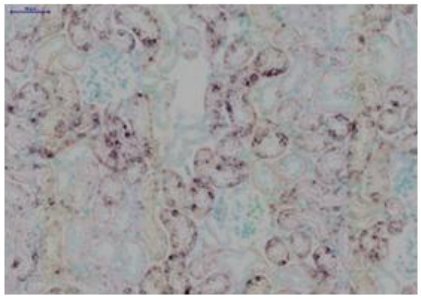
The *Clr-d* transcripts in the eye were found to be expressed in the epithelial cells of the sclera as well as areas around the optical nerve (**Figure 10**).

Clr-g is detected in the white pulp area of the spleen. It is also detected in the thymus and lymph node but at lower levels compared to *Clr-b.* Furthermore, *Clr-g* is expressed in non-lymphoid tissues. It is expressed in the epithelial lining of the intestine and some cells within the lamina propria, with non-specific staining in the goblet cells. It is also detected in the lung epithelial cells which are mainly the type II alveolar epithelial cells and a few bronchial epithelial cells (**Figure 11**).

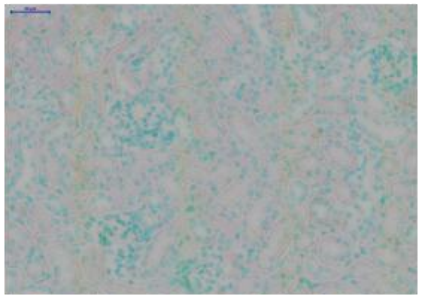
Figure 8: *Clr-f* is expressed in the kidney tubular epithelial cells. *In situ* hybridization was performed to detect *Clr-f* gene in the kidney. 4µm thick kidney paraffin-embedded sections were permeabilized and hybridized with 500ng/ml of *Clr-f* antisense and sense (negative control) probes labeled with digoxigenin. Color development was performed using anti-DIG-AP conjugated antibody followed by adding NBT/BCIP substrate, which forms a purple precipitate on the slides. H&E staining for the kidney is shown to visualize the kidney structure. The scale bar indicates 50 µm.

*Clr-f*_kidney

anti sense



sense



H&E

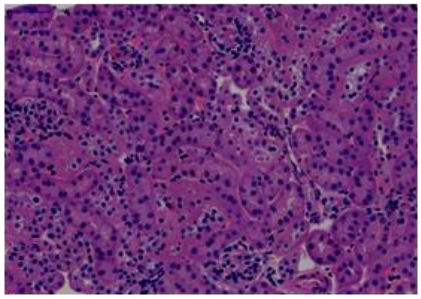
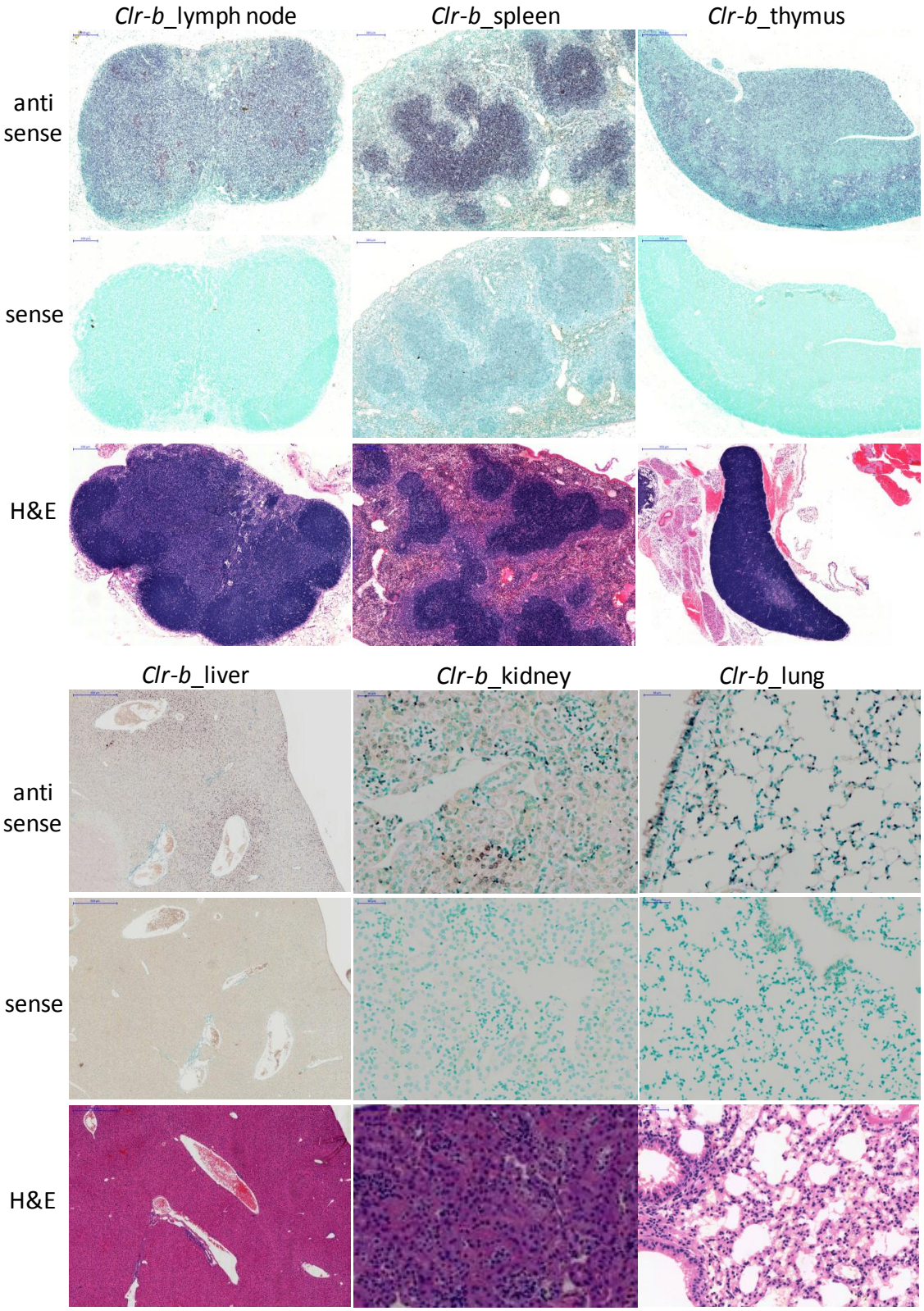


Figure 9: *Clr-b* is broadly expressed in different tissues. *In situ* hybridization was performed to detect the *Clr-b* gene in different tissues from adult B6 mice. Inguinal lymph node, spleen, thymus, liver, kidney, lung, skeletal muscle, heart were fixed in 10% neutral buffered formalin at room temperature following dissection. 4μm thick paraffin-embedded tissue sections were permeabilized and hybridized with 500ng/ml of *Clr-b* antisense and sense (negative control) probes labeled with digoxigenin. The skeletal muscle section was hybridized with *Clr-g* antisense probe to test probe specificity. Color development was performed using anti-DIG-AP followed by adding NBT/BCIP substrate, which forms a purple precipitate on the slides. H&E staining for each type of tissues is shown to visualize the structure of each tissue. The scale bar indicate 200 μm in the inguinal lymph node and spleen, 500 μm in the thymus and liver, 50 μm in the kidney, lung, skeletal muscle and heart.


*Clr-b*_lymph node
*Clr-b*_spleen
*Clr-b*_thymus
anti sense
sense
H&E
*Clr-b*_liver
*Clr-b*_kidney
*Clr-b*_lung
anti sense
sense
H&E

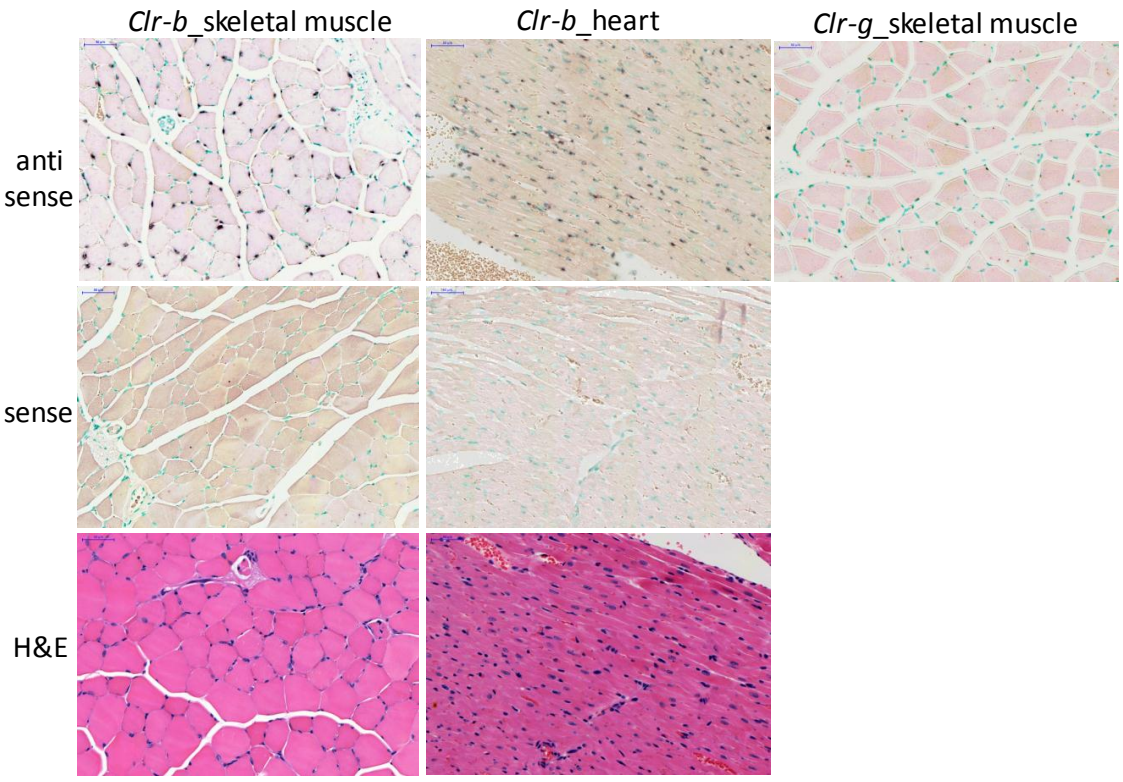
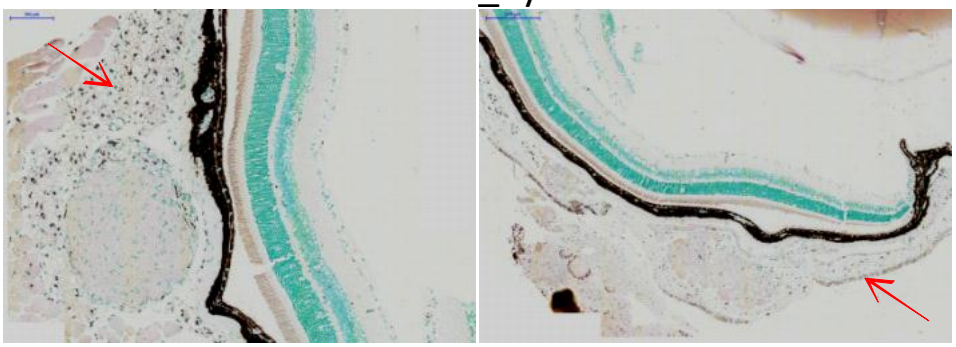

*Clr-b*_skeletal muscle
*Clr-b*_heart
*Clr-g*_skeletal muscle
anti sense
sense
H&E

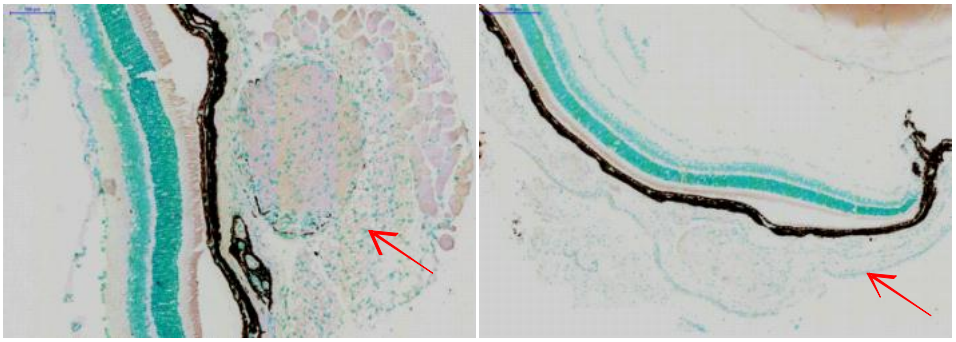
Figure 10: *Clr-d* transcript is detected in the eye. *In situ* hybridization was performed to detect the *Clr-d* gene in the eye. 4μm thick paraffin-embedded sections were permeabilized and hybridized with 500ng/ml of *Clr-d* antisense and sense (negative control) probes labeled with digoxigenin. Color development was performed using anti-DIG-AP followed by adding NBT/BCIP substrate, which forms a purple precipitate on the slides. H&E staining for the mouse eye is shown to visualize the structure of the eye. The scale bar indicates 200 μm in the sclera epithelial cell section and 100 μm in the optic nerve section.

*Clr-d*_eye

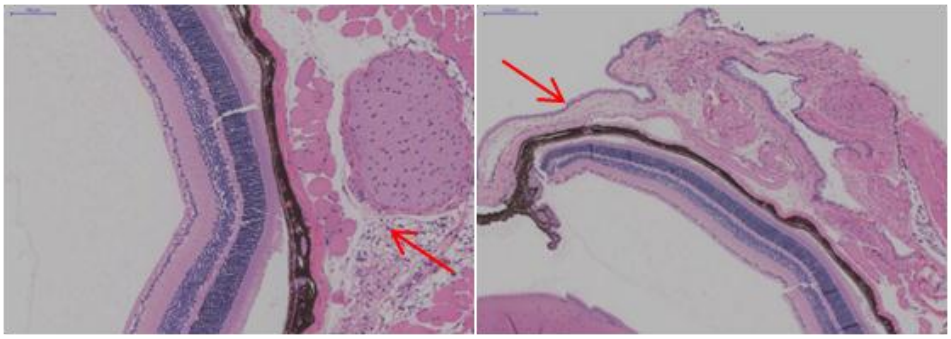
anti sense



sense



H&E



H&E

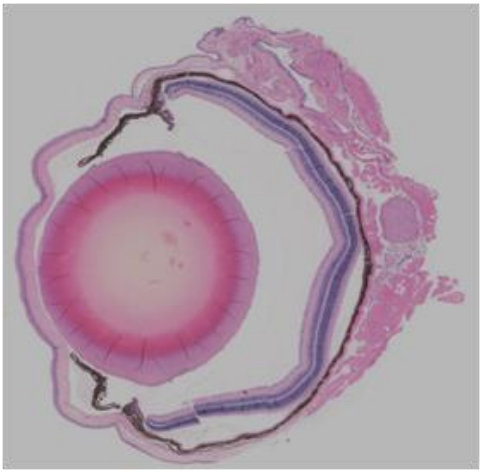
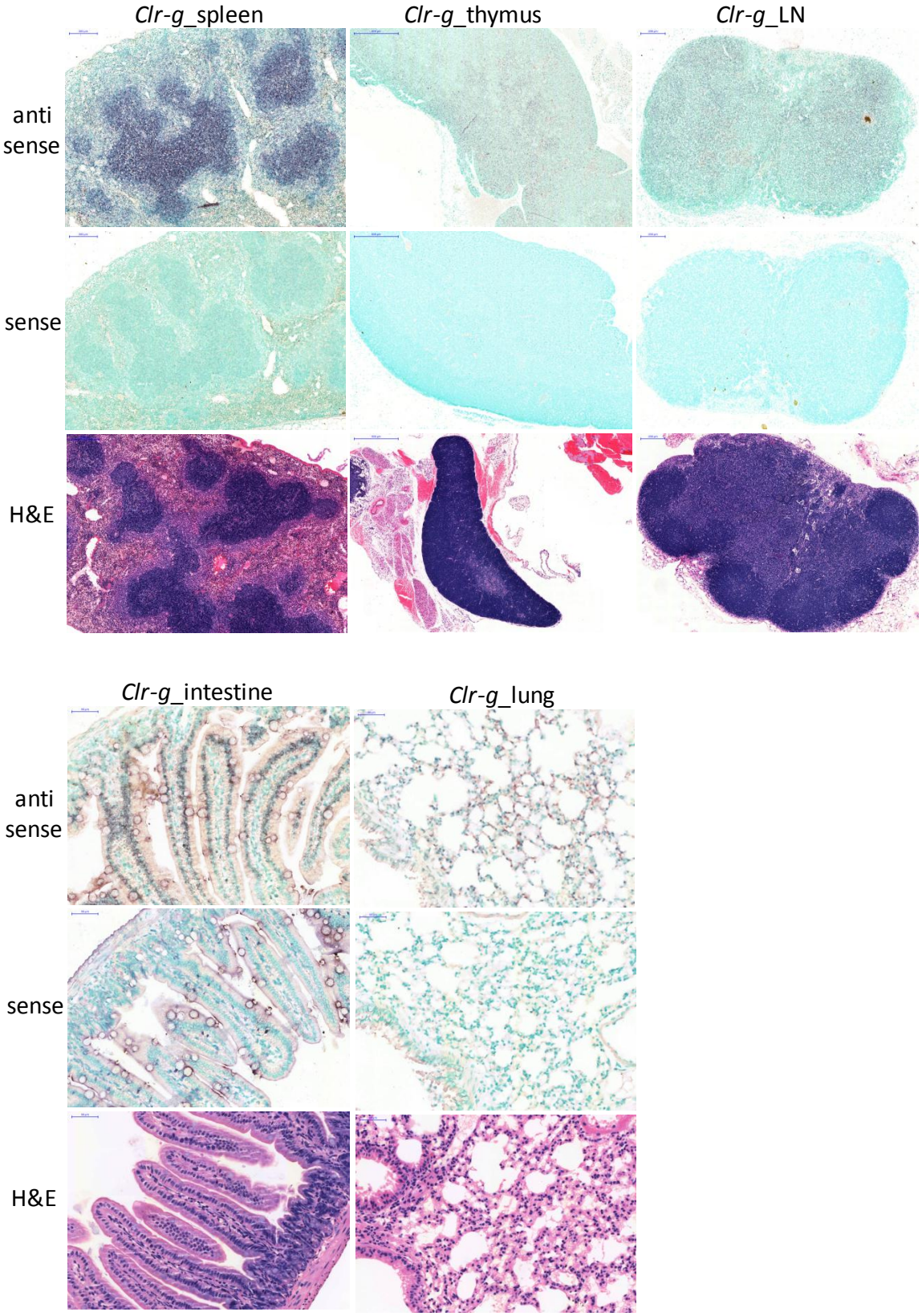


Figure 11: *Clr-g* is expressed in both lymphoid and non-lymphoid tissues. *In situ* hybridization was performed to detect the *Clr-g* gene in different tissues including the inguinal lymph node, spleen, thymus, intestine and lung. 4μm thick paraffin-embedded sections were permeabilized and hybridized with 500ng/ml of *Clr-g* antisense and sense (negative control) probes labeled with digoxigenin. Color development was performed using anti-DIG-AP followed by adding NBT/BCIP substrate, which forms a purple precipitate on the slides. H&E staining for each type of tissues is shown to visualize the structure of each tissue. The scale bar indicates 200 μm in the inguinal lymph node and spleen, 500 μm in the thymus, 50 μm in the intestine and lung.


*Clr-g*_spleen
*Clr-g*_thymus
*Clr-g*_LN
anti sense
sense
H&E
*Clr-g*_intestine
*Clr-g*_lung
anti sense
sense
H&E

4 Discussion

The NKR-P1 receptor family was the first NK cell receptor to be discovered over thirty years ago (137); however, they have not been well characterized in terms of genomic and functional studies compared to the related Ly49 receptor family. In order to gain understanding of the NKR-P1 receptor family, genetic and expression studies were performed.

4.1 Genetic conservation of the *Nkrp1-Clr* gene cluster

The *Nkrp1-Clr* gene cluster is highly conserved in the three mouse stains studied, including B6, 129S6 and BALB/c. BAC clone sequencing reveals that the *Nkrp1-Clr* gene cluster from 129S6 and BALB/c mouse strains are highly similar to the B6 counterparts. The gene number, genomic organization and gene orientation for the *Nkrp1-Clr* gene cluster are identical among three mouse strains (**Figure 3 and 5**). However, it may be possible that there are other mouse strains that have diverse *Nkrp1-Clr* gene clusters compared to the mouse strains studied here. For example, the NOD mouse strain falls into a different subgroup from the mouse strains studied above as were determined by the RFLP analysis of *Nkrp1* genes and aCGH analysis by others (134).

Nkrp1 and *Clr* genes have low polymorphism at the allelic level (**Table 3**). Most of the NKR-P1 receptors and Clr ligands share 100% identical protein sequences between the 129S6 and BALB/c mice while they diverge from the B6 counterparts. Notably, the Clr-c and NKR-P1F proteins sequence are 100% conserved between the B6 and BALB/c mice, however, they are different from that of the 129S6 mice. Clr-a is 99% identical in BALB/c and B6 mice, and it is 97% identical between 129S6 and B6/BALB/c. As such, the *Nkrp1* and *Clr* genes from the 129S6 and BALB/c are suggested to be more closely related, whereas the B6 counterpart shows relative divergence. This is in agreement with previous studies

showing that the 129S1 and BALB/c *Nkrp1* cluster belong to the same subgroup as determined by the aCGH and RFLP analysis, whereas the B6 counterpart falls into another group (134). Our southern blot analysis of RFLP patterns for *Clr* genes confirmed that *Clr* genes are highly conserved as well. The twelve mouse strains studied seem to fall into three groups according to their RFLP patterns for *Clr* genes (**Figure 4**). Group one includes 129P3/J, DBA/2J, 129S1/SvImJ, A/J, CBA/J, C3H/HeJ and BALB/cJ mouse strains; group two includes C57BL/10J, C57L/J, FVB/NJ and C57BL/6J mouse strains; group three includes NOD/Lt mouse strain. For *Nkrp1s*, previous studies have shown that the DBA/2J, 129S1/SvImJ, A/J, CBA/J, C3H/HeJ and BALB/cByJ mouse strains fall in the same subgroup (134). The NOD/ShiLt/J mouse strain belongs to a distinct subgroup (134). Finally, the C57L/J and FVB/NJ mouse strains belong to the same subgroup while the C57BL/6J mouse strain falls into a different subgroup (134).

Overall, the allelic polymorphism analyses for *Nkrp1* and *Clr* genes shows focused diversity on *Clr-c*, *Nkrp1b* and *Nkrp1c* (**Table 3**). There may be multiple reasons for such focused divergence. One of the possible explanations is the selective pressure to evolve under constant pathogen challenge.

The expression level of Clr-c, the ligand for the activating receptor NKR-P1F, must be strictly controlled in the host to avoid autoimmunity; however, Clr-c expression, on infected cells, may be upregulated upon viral infection making them susceptible to elimination by NK cells. To escape NK cell response, the virus may adapt to downregulate surface Clr-c expression. This has been seen in the case of infection with HCMV and MCMV, both of which interfere with the surface expression of NKG2D ligands (138, 139). In response to this,

the host may accumulate mutations in Clr-c to avoid virus targeting while retaining its interaction with NKR-P1F receptor in order to activate NK cells.

NKR-P1B, the inhibitory NK cell receptor, interacts with Clr-b to inhibit NK cell function. It is possible that some viruses utilize mimics of Clr-b to escape NK cell killing. This has been shown in the case of RCMV which utilizes a mimic Clr-b to inhibit NK cell activation through interactions with rat NKR-P1B receptor (140). The polymorphic variants of the NKR-P1B receptor would make the host unable to engage with the virus Clr-b mimic, meanwhile maintaining the interaction with self-Clr-b to protect healthy host cells from killing. A previous study has shown that besides the above strategy, the host has evolved an activating receptor from an inhibitory receptor which specifically recognizes the viral decoy of the inhibitory receptor ligand. In B6 mice, the activating receptor Ly49H is thought to originate from the inhibitory Ly49I. Ly49H recognizes m157, which is encoded by MCMV, and is the mimic of self-MHC interacting with the Ly49I receptor. However, in 129 mice, Ly49H is not present, and as a result, the 129 mouse strain is susceptible to MCMV infection (11, 93).

NKR-P1C is an activating receptor. The physiological ligand of the NKR-P1C receptor is unknown, making it difficult to understand the NKR-P1C allelic polymorphism. Nonetheless, NKR-P1C may be able to recognize a virus derived Clr protein. This Clr protein is possibly able to interact with an inhibitory NK cell receptor and may evolve rapidly in order to evade NK cell killing. In response to this, the host NKR-P1C receptor must evolve to retain the interaction with the virus Clr protein. Alternatively, the NKR-P1C receptor may recognize a Clr protein that is present in mice, in which case the interaction between the receptor-ligand pair would be strictly controlled at the protein expression level to prevent

autoimmunity. It is also possible that NKR-P1C recognizes a Clr protein whose expression is induced on abnormal cells such as tumor and virus-infected cells but not on healthy cells. Finally, NKR-P1C could possibly interact with non-Clr proteins and these ligands may be under rapid evolution, thus resulting in the polymorphism of the NKR-P1C receptor to retain the interaction.

The conserved nature of the *Nkrp1-Clr* gene cluster is in contrast to the highly diverse *Ly49* gene cluster. The *Ly49* gene cluster shows high genetic diversity in gene content and gene organization in different mouse strains; additionally, different mouse strains have high allelic polymorphism for individual *Ly49* genes (89-91). The ligands for the Ly49 receptors are the highly polymorphic MHC-I molecules, which are encoded by genes present on another chromosome, thus allowing for independent segregation. Ly49 receptors have evolved in order to maintain interaction with equally diverse ligands (108). Moreover, there is evidence indicating the extreme plasticity of *Ly49* gene clusters may be due to high percentage of the LINE1 repeat elements within the gene cluster, which may allow for frequent non-homologous recombination events and gene evolution (92). Our study and others show that the genes encoding the NKR-P1 receptors and the Clr ligands are intermingled within the same genomic region, allowing for co-segregation of the genes and protection of the receptor-ligand interaction (108). Interestingly, the analysis of repeat elements show that the percentage of LINE1 repeat elements within the *Nkrp1-Clr* gene cluster is close to that within the whole genome and significantly lower than that within the *Ly49* gene cluster (**Table 2**). This may indicate limited homologous recombination within the *Nkrp1-Clr* gene cluster, leading to gene conservation, and confirms the hypothesis that a higher percentage of LINE1 leads to higher gene polymorphism in *Ly49* gene cluster. An

unexpected finding was the high percentage of LTR elements within the *Nkrp1-Clr* gene cluster (26%), which is more than twice the percentage within the *Ly49* gene cluster (12%) and the genome wide average (10%) (**Table 2**) (132, 141). LTR elements are derived from ERVs in mice (141). The ERV_classII subgroup of LTR elements accounts for the high percentage of LTR elements within the *Nkrp1-Clr* gene cluster. It remains unknown why there is significantly higher LTR elements within the *Nkrp1-Clr* gene cluster.

4.2 *Nkrp1* and *Clr* gene expression profile

Due to the lack of specific antibodies for most of the NKR-P1 and Clr molecules, we determined *Nkrp1* and *Clr* gene transcripts expression in different tissues by RT-PCR and *in situ* hybridization studies. For confirmation the expression data were compared with microarray data performed by others. The drawbacks of this study include, first, RT-PCR may not detect splicing variants of certain genes, and thus a negative RT-PCR result may not be indicative of the absence of possibly functional splicing variants. Second, all the RT-PCR analyses were performed on tissues or organs collected from adult mice. Whether *Nkrp1* and *Clr* gene transcripts are differentially expressed during developmental stages remains unknown. Third, some of the microarray data is not consistent with the RT-PCR results. The microarray analysis sometimes results in false positive or false negative results due to different standards used when taking into account the background noise and the normalization process. Additionally, the probes designed for detecting certain genes may not be as specific and could cross-react with other closely related genes. Fourth, it is possible that the positive signals detected by *in situ* hybridization are from the interaction of the probe with non-functional splicing variants of the gene or with some gene fragments that have non-specifically bound to the probe. The probes may also have non-specific cross-reaction with

other members of the gene family. Finally, even if the complete functional transcript of a certain gene is expressed in the tissue, the corresponding protein is not necessarily translated. However, whether the Clr proteins are expressed can only be determined once the corresponding antibodies are available. Nonetheless, our expression studies reveal an interesting pattern of expression of *Nkrp1* and *Clr* genes not only in lymphoid but also in the epithelial cells of the intestine, kidney, eye and lung, the myocytes of the heart and skeletal muscle, and possibly some endothelial cells. This is in contrast to more widely expressed MHC-I molecules. MHC-I molecules are widely expressed in all nucleated cells, which makes it a sign of health as abnormal expression of MHC-I on these cells would be recognized by NK cells (6). These unhealthy cells will eventually be lysed by NK cells.

Amongst the *Clr* genes, *Clr-b* is broadly expressed in most hematopoietic cells with extended expression in non-hematopoietic cells such as myocytes and lung epithelial cells, indicating novel functions of mouse NK cells in these tissues (**Figure 9**). Broad expression of the *Clr-b* transcript in various tissues makes it an ideal candidate as a signal of health used by NK cells, similar to the widely-expressed MHC-I molecules. Abnormal cell surface expression of Clr-b induced by bacteria and viral infection, tumor transformation or stress, such as injuries or oxidation, will be recognized by NK cells resulting in elimination of these cells.

Rat *Clr11* is the homolog of mouse *Clr-b* in terms of the relative genomic distribution since both are close to the *CD69* gene (92). Clr11 binds to the inhibitory receptor NKR-P1B in rat (121), thus, function is conserved with the mouse NKR-P1B/Clr-b receptor-ligand counterparts. The transcript expression study of the *Clr11* gene shows that it is highly expressed in B cells, NK cells, spleen, thymus, liver, heart, muscle and kidney (120), which

is similar to our transcript expression results of mouse *Clr-b*. Thus, Clr-b and Clr11 may have similar functions apart from being evolutionarily conserved in genome organization.

Human NKR-P1A receptor and its ligand LLT1 have high sequence homology with mouse NKR-P1B and Clr-b, respectively (92). The interaction between human NKR-P1A and LLT1 inhibits NK cell activity which resembles the NKR-P1B/Clr-b interaction in mice (115). LLT1 expression is restricted to activated DCs, B cells, T cells and NK cells (118). However, *LLT1* transcripts are detected in tissues such as heart and skeletal muscle based on previous microarray studies (135, 142). Thus, it would be interesting to study whether LLT1 protein is expressed in the aforementioned tissues. This work would aid in the discovery of novel functions of human NK cells.

An interesting finding was the constitutive expression of *Clr-f* transcripts in different parts of the intestine from three different mouse strains (**Figure 7**), indicating that Clr-f may be present in structural cells of the intestine and may be functionally conserved in different mouse strains. High expression of *Clr-f* in the epithelial lining of the intestine and kidney tubules may function as a signal of health used by NK cells (**Figure 7 and 8**). Following bacterial or viral infection, or under stress conditions such as injury, *Clr-f* may be downregulated. As such, NK cells would recognize the abnormal *Clr-f* expression and kill these unhealthy cells. In order to validate this, *in situ* hybridization can be performed to check *Clr-f* expression on intestine sections from mice infected with pathogenic gut-specific bacteria and viruses, or on kidney sections from mice with certain injuries. Rat *Clr9* is the orthologue of the mouse *Clr-f* gene (121). However, no transcript expression data for rat *Clr9* is available, making it difficult to assess functional conservation to mouse Clr-f.

Clr-d transcript expression is detected in the eye (**Figure 10**). It is the ligand for NKR-P1F. The NKR-P1F receptor has a positive charge in the transmembrane domain and does not possess an ITIM in its intracellular domain, making it a potential activating receptor (6). However, NKR-P1F is not able to induce cytokine production or cytotocixity in NK cells when cross-linked with specific mAb (143). Therefore, whether NKR-P1F acts as an activating receptor or not remains unknown. On the other hand, the inhibitory NKR-P1G receptor also interacts with Clr-d (109). This interaction may be responsible for preventing over-activation of NK cells and also any other lymphocytes that express NKR-P1G receptor in the eye, which is a highly immune privileged site wherein any inflammation is undesirable.

Similar to Clr-d, Clr-g is the ligand for both NKR-P1F and NKR-P1G receptors (109). The *Clr-g* transcript is detected in both lymphoid and non-lymphoid organs (**Figure 11**). Therefore, Clr-g may also act as a signal of health used by NK cells. It must be noted that *Clr-g* expression in the thymus and lymph node is weaker than *Clr-b* expression (**Figure 9 and 11**). The weak signal observed in these tissues could be due to the interaction between the *Clr-g* probe and the *Clr-b* mRNA since *Clr-g* and *Clr-b* are clustered closely together in phylogenetic analyses (109).

Mouse *Clr-a* is only detected in the intestine in our RT-PCR analysis, but this result agrees with the available microarray data (**Table 4A**). Compared to this, the rat *Clr10,* which is potentially an orthologue of mouse *Clr-a*, is highly expressed in the thymus, and moderately expressed in the spleen and liver (120, 121). Whether rat *Clr10* is expressed in the intestine remains to be investigated.

The restricted expression pattern of *Clr* genes in different tissues suggest novel and diverse function of NK cells in different tissues. Whether Clr proteins play a role in

regulating NK cells functions in pathogenic conditions remain to be fully determined. In order to gain further understanding into the role of Clr proteins following viral infection, tumor immune-surveillance or stress conditions, analysis of *Clr* expression should be performed in different tissues under such conditions. Furthermore, it would be interesting to study the promoter sequences of the genes and whether there is a correlation between the presence of a tissue-specific promoter and corresponding tissue-specific expression. Finally, *in situ* hybridization studies for tissue and cell type-restricted expression of *Nkrp1* genes is required to determine whether NKR-P1 receptors are expressed in a coordinate fashion with their Clr ligands.

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Contribution of Collaborators

Paraffin tissue slides used in *in situ* hybridization experiments were prepared by members of the Histology Core Facility at Roger Guindon Hall, including the following steps: tissue embedding into paraffin blocks, section cutting and mounting. H&E staining and coverslip were also performed by them. BAC clones generated from the 129S6 and BALB/c genomic library which contain the *Nkrp1* and *Clr* genes were sent to Genome Quebec Innovation Centre (Montreal) and The Centre for Applied Genomics, SickKids Hospital (Toronto) for sequencing by Dr. Munir Rahim. Cell sorting of the intestinal epithelial cells in Figure 7b was performed by the Ottawa Hospital Research Institute Flow Cytometry Facility. Plasmids were sent to the Ottawa Hospital Research Institute DNA Sequencing Facility for sequencing**.**

Appendices

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- Flow cytometry and cell sorting
- Southern and northern blotting
- Cytotoxicity assay

Publications

- P. Chen, S. Bélanger, O. A. Aguilar, **Q. Zhang**, A. St-Laurent, M. M. A. Rahim, A. P. Makrigiannis, and J. R. Carlyle, 'Analysis of the Mouse 129-Strain Nkrp1-Clr Gene Cluster Reveals Conservation of Genomic Organization and Functional Receptor-Ligand Interactions Despite Significant Allelic Polymorphism', ***Immunogenetics***, 63 (2011), 627-40.
- **Qiang Zhang**, Mir Munir A. Rahim, Megan M. Tu, David Allan, Simon Belanger, Elias Abou-Samra, Harman Sekhon, Todd Fairhead, Haggag S. Zein, Stephen K. Anderson,

James R. Carlyle, and Andrew P. Makrigiannis, '*Nkrp1-Clr* gene cluster sequence and expression analyses in the mouse reveals conservation of organ-specific MHC-independent immunosurveillance', PLoS ONE. (Submitted)

Presentations and Posters

- Type: Poster
  Event: International graduate student immunology conference, Harvard University, Boston, MA, USA April 27-28, 2012
  Title: Genetic and expression analyses of the *Nkrp1-Clr* gene cluster
- Type: Oral
  Event: University of Ottawa, Department of Biochemistry, Microbiology and Immunology, Seminar Day February 2012
  Title: Genetic and expression analyses of the *Nkrp1-Clr* gene cluster
- Type: Poster
  Event: University of Ottawa, Department of Biochemistry, Microbiology and Immunology, Poster Day May 2011
  Title: Genetic and expression analyses of the *Nkrp1-Clr* gene cluster