Elucidation of ligand and function for the mouse plasmacytoid dendritic cell receptor Ly49Q

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ABSTRACT

Plasmacytoid dendritic cells (pDCs) are especially suited for initiating immune responses against viruses because they are the most potent producers of type I interferon (IFN). This property of controlling viral infections is accomplished by their selective expression of toll-like receptor (TLR)-7 and TLR-9, which recognize viral single-stranded RNA (ssRNA) and unmethylated cytosine-phosphate-guanine (CpG)-containing double-stranded DNA (dsDNA), respectively. Through the secretion of Type I IFN, pDCs can modulate natural killer (NK) cell cytotoxicity, myeloid DC (mDC) activation, B cell maturation and T cell priming. Mouse pDCs express Ly49Q, a C-type lectin-like immunoreceptor tyrosine-based inhibitory motif (ITIM) -containing receptor that binds to the classical major histocompatibility complex class I molecule (MHC-Ia), H-2K^b. Prior to our studies, both the ligand and function of Ly49Q on pDCs was unknown.

We used reporter cell analysis to demonstrate that a high-affinity ligand for Ly49Q is present on H-2^b, but not H-2^d, H-2^k, H-2^q, or H-2^a-derived tumour cells and normal cells *ex vivo*. The ligand is peptide-dependent and MHC-Ia-like, as revealed by its functional absence on cells deficient in TAP-1, β_2m, or H-2K^{b}D^b expression. Furthermore, Ly49Q is specific for H-2K^b, as the receptor binds peptide-loaded H-2K^b but not H-2D^b complexes, and Ly49Q recognition can be blocked using anti-K^b but not anti-D^b mAb. These results demonstrate that Ly49Q efficiently binds H-2K^b and suggest that pDC function is regulated by classical MHC-Ia molecules.

To characterize the implications of Ly49Q: H-2K^b interaction, we utilized the Ly49Q-deficient mice previously generated in our lab. *Ex vivo* pDCs stimulated with CpG resulted in enhanced IFN-α and IL-12 production. Blocking with soluble Ly49Q or H-2K^b mAb abrogated cytokine production, while receptor cross-linking with platebound anti-Ly49Q and recombinant H-2K^b increased cytokine production. Sera obtained from CpG injected Ly49Q-deficient mice and supernatant obtained from CpG stimulated Ly49Q-deficient pDCs displayed reduced cytokine production.

Taken together, we conclude that Ly49Q recognition of H-2K^b is crucial for pDC cytokine production. Insight into human pDC immune response against transformed and pathogen infected cells can be acquired from characterizing the Ly49Q ligand and receptor in the mouse model.

RESUME

Les cellules dendritiques plasmacytoïdes (pDCs) sont très efficaces pour initier une réponse immunitaire contre les virus parce qu'elles sont des productrices efficaces d'interféron (IFN) de type I. Cette habileté à contrôler les infections virales est accompagnée par une expression sélective des récepteurs Toll-like (TLR)-7 et TLR-9 qui reconnaissent, en ordre, l'ARN viral à simple brin et l'ADN à double brin contenant des séquences cytosine-phosphate-guanosine (CpG) non-méthylées. Par la sécrétion d'IFN de type I, les pDCs peuvent moduler la cytotoxicité des cellules natural killer (NK), l'activation des DC myéloïdes (mDCs), la maturation des lymphocytes B et l'activation des lymphocytes T. Les pDCs murines expriment le récepteur Ly49Q, une lectine de type C contenant une séquence de type immunoreceptor tyrosine-based inhibitory motif (ITIM) qui reconnaît une molécule classique du complexe majeur d'histocompatibilité (CMH de classe Ia), H-2K^b. Avant nos études, le ligand et la fonction de Ly49Q chez les pDCs étaient inconnus.

Nous avons utilisé un test de cellules ayant un gène rapporteur pour démontrer qu'un ligand pour Ly49Q est présent sur des cellules tumorales et des cellules isolées *ex vivo* d'origine H-2^b, mais pas d'origine H-2^d, H-2^k, H-2^q, ou H-2^a. Le ligand dépend de la présence de peptides et est une molécule CMH de type Ia puisque le ligand est absent de cellules déficientes pour l'expression de TAP-1, β_2m, ou d'H-2K^{b}D^b. De plus, Ly49Q est spécifique pour H-2K^b, puisque le récepteur se lie à H-2K^b couplé à un peptide et non à un complexe de peptide et d'H-2D^b et la reconnaissance par Ly49Q peut être bloquée par un anticorps pour

H-2K^b mais pas pour H-2D^b. Ces résultats démontrent que Ly49Q se lie efficacement à H-2K^b et suggèrent que la fonction des pDCs est contrôlée par des molécules classiques CMH de type Ia.

Afin de caractériser les implications de l'interaction entre Ly49Q et H-2K^b, nous avons utilisé la souris déficiente pour Ly49Q précédemment générée par notre laboratoire. Des pDCs isolés *ex vivo* stimulées avec CpG produisent des quantités élevées d'IFN-α et d'IL-12. La production de cytokines peut être bloquée à l'aide d'anticorps pour Ly49Q ou H-2K^b tandis que la ligation de Ly49Q à l'aide d'un anticorps ou d'H-2K^b recombinant augmente la production de cytokines. Du sérum isolé de souris déficientes en Ly49Q injectées avec du CpG et du milieu de culture de pDCs déficients en Ly49Q stimulés avec du CpG démontrent une production diminuée de cytokines.

Pour résumer, nous concluons que la reconnaissance d'H-2K^b par Ly49Q est cruciale pour la production de cytokines par les pDCs. Une meilleure compréhension de la réponse immunitaire des pDCs humains contre les cellules transformées et infectées par des pathogènes peut être obtenue par l'étude du récepteur Ly49Q et de son ligand dans le modèle murin.

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TABLE OF CONTENTS

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The following original results and concepts are presented in this thesis:

The identification of H-2K^b as the physiological ligand for the mouse pDC receptor Ly49Q.

We utilized a reporter cell-based assay and *ex vivo* pDCs to demonstrate the direct recognition of H-2K^b by Ly49Q. This represents the first finding of pDC recognition of MHC class I molecules. Similar to NK and T cells, pDC recognition of the presence or absence of MHC has important implications for viral and cancer research.

The characterization of Ly49Q function on pDCs.

Using Ly49Q-deficient mice, we demonstrated that Ly49Q is crucial for modulating pDC function, including the TLR-9 triggered production of IFN-α and IL-12. This represents the first finding of an ITIM-containing C-type lectin like receptor on pDCs that positively regulates IFN-α and IL-12 production.

CONTRIBUTION OF AUTHORS

Chapter 1 is a survey of the literature and provides the reader with the necessary background information to understand and critique this thesis. Chapter 4 is the authors's discussion on research conclusions, implications and opportunities for future research. Manuscript-based chapters include Chapters 2 and 3. Both Chapters 2 and 3 are research papers, for which Dr Andrew Makrigiannis, as supervisor, conceived the studies, provided insight throughout, prepared and edited the manuscripts. Lee-Hwa Tai carried out all the experiments associated with Chapter 2, the great majority of the experiments associated with Chapter 3, conceived the studies for Chapter 3, and prepared and edited a portion of the manuscript for Chapter 3. Detailed contributions are outlined below.

Chapter 2:

Tai L-H, Goulet M-L, Bélanger S, Troke AD, St-Laurent AG, Mesci A, Toyama-Sorimachi N, Caryle JR, Makrigiannis AP. *Recognition of H-2K^b by Ly49Q suggests a role for class Ia MHC regulation of plasmacytoid dendritic cell function*. Molecular Immunology, 2007, 44(10): p.2638-46.

All laboratory experiments were carried out by Lee-Hwa Tai. Marie-Line Goulet and Angela Troke provided experimental training and helpful discussion. Simon Belanger assisted in mice dissection. Aaron St-Laurent stained *ex vivo* pDCs with H-2K^b tetramer. Aruz Mesci and Dr. James Carlyle assisted in the creation of BWZ reporter cells. Dr. James Carlyle also provided experimental training, helpful insight and assisted with the final editing of the manuscript. Dr. Noriko Toyama-Sorimachi provided the human Ly49Q-Ig fusion protein and Figure 3C.

Dr. Andrew Makrigiannis conceived the experiments, prepared the manuscript and provided insight and guidance throughout.

Chapter 3:

Tai L-H, Goulet M-L, Bélanger S, Toyama-Sorimachi N, Fodil-Cornu N, Vidal SM, Troke AD, McVicar DW, Makrigiannis AP. *Positive regulation of plasmacytoid dendritic cell function via Ly49Q recognition of class I MHC*. Journal of Experimental Medicine, 2008, 205(13): p.3187-99.

Lee-Hwa Tai and Marie-Line Goulet contributed equally to this paper.

Lee-Hwa Tai performed the experiments for the following figures: Figures 1, 2E, 3C, 4A-D, 5, 6A, 7A and B, 8A and B; conceived the experiments; genotyped mice; prepared and edited the manuscript.

Marie-Line Goulet performed the experiments for the following figures: Figures 2A-D, 3A and B, 6B and C, 7C, 8C; conceived the experiments; assisted in the initial creation of the Ly49Q-deficient mice; and genotyped mice.

Simon Bélanger performed and assisted with the experiments for the following figures: Figures 1E and F; genotyped mice; provided initial ELISA training, offered insight throughout.

Dr. Noriko Toyama-Sorimachi provided anti-Ly49Q NS-34 antibody and insight.

Dr. Nassima Fodil-Cornu and Dr. Silvia Vidal provided MEFS and training with MCMV plaque assays.

Angela Troke determined TLR-9 expression by RT-PCR and provided training and assistance with MCMV plaque assays.

Dr. Daniel McVicar provided helpful insight.

Dr. Andrew Makrigiannis conceived the experiments; prepared and edited the manuscript; provided insight and guidance throughout.

Other related manuscripts for which I have made valuable contributions during my doctoral program, but that are not included as part of this thesis, are as follows:

Carlyle JR, Mesci A, Fine JH, Chen P, Bélanger S, **Tai L-H**, Makrigiannis AP. *Evolution of the Ly49 and Nkrp1 recognition systems*. Seminars in Immunology. 2008, 20(6):321-30.

Bélanger S, **Tai L-H**, Anderson S, Makrigiannis AP. *Ly49 cluster sequence in a mouse model of diabetes: an expanded repertoire of activating receptors in the NOD genome*. Genes and Immunity. 2008, 9(6):509-21.

Colina R, Costa-Mattioli M, Dowling RJO, Jaramillo M, **Tai L-H**, Breitbach CJ, Martineau Y, Larsson O, Rong L, Svitkin YV, Makrigiannis AP, Bell J, Sonenberg N. *Translational control of the innate immune response via IRF-7*. Nature, *2008,* 451(7185):323-8.

Carlyle JR, Mesci A, Ljutic B, Bélanger S, **Tai L-H**, Rouselle E, Troke AD, Proteau M-F, Makrigiannis AP. *Molecular and Genetic Basis for Strain-Dependent NK1.1 Alloreactivity of Mouse NK Cells*. Journal of Immunology, 2006, 176: 7511-7524.

LIST OF ABBREVIATIONS

APC: Antigen presenting cell
β_2-m: β_2-microglobulin
BDCA-2/-4: Blood dendritic cell antigen-2/-4
BM: Bone marrow
Bp: Base pairs
BST-2: Bone marrow stromal cell antigen-2
BSA: Bovine serum albumin
B6: C57BL/6 mice
CCR: Cysteine:cysteine N terminus chemokine receptor
CD: Cluster of differentiation
CHO: Chinese hamster ovary
CpG ODN: Cytosine-phosphate-Guanine oligodeoxynucleotides
CMV: Cytomegalovirus
CTL: Cytotoxic T lymphocytes
CTLD: C-type lectin-like domain
CTTP: Cytoplasmic transductional-transcriptional processor CTTP
CXCR: Cysteine:amino acid:cysteine N terminus chemokine receptor
DAP-10/12: DNAX activation protein-10/-12
DC: Dendritic cell
DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
dsDNA; double stranded DNA
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
ES cells: Embryonic stem cells
FBS: Fetal bovine serum
FcR: Fragment cystalizable receptor
FACS: Fluorescence activated cell sorting
Flt3L: Fms-like tyrosine kinase 3 -ligand
GM-CSF: Granulocyte macrophage colony growth stem cell factor
GPI: Glycosylphosphatidyl-inosinol luminal
HBD-1: Human beta-defensin 1 (HBD-1)
HEV: High endothelial venule
HIV: Human immunodeficiency virus
HMGB1: High-mobility group box 1
HNSCC: Head and neck squamous cell carcinoma
HSV: Herpes simplex virus
Id2/3: Inhibitors of differentiation 2/3
IFN: Interferon
IgH : Ig heavy chain
IL: Interleukin
ILT7: Immunoglobulin-like transcripts receptor
IP: Intraperitoneal
IPC: Type I interferon producing cell

IPS-1: IFN-β promoter stimulator-1
IRAK: IL-1 receptor associated kinase
IRCM: Institut de recherches cliniques de Montreal
IRES-GFP: Internal ribosomal entry site – green fluorescent protein
IRF: Interferon regulatory factor
ITAM: Immunoreceptor tyrosine-based activating receptor
ITIM: Immunoreceptor tyrosine-based inhibitory receptor
IV: Intravenous
KIR: Killer cell Ig-like receptor
KO: Knockout
LAIR: Leukocyte-associated Ig-like receptor
LCMV: Lymphocytic choriomeningitis virus
LIR: Leukocyte Ig-like receptor
LN: Lymph node
LPS: Lipopolysaccharide
mAb: Monoclonal antibody
ManLAM: Lipoarabinomannans
MAPK: Mitogen-activated protein kinase
MCMV: Mouse cytomegalovirus
MDA-5: Melanoma differentiation-associated gene 5
mDC: Myeloid dendritic cell
MDDC: Monocyte derived DC
MFI: Mean fluorescence intensity
MHC: Major histocompatibility complex
MHC-Ia: Classical major histocompatibility complex molecules class Ia
MHC-Ib: Nonclassical major histocompatibility complex molecules class Ib
MLN: Mesenteric lymph node
mPDCA-1: Mouse plasmacytoid dendritic cell antigen 1
MyD88: Myeloid differentiation primary-response gene 88
NF-κβ: Nuclear factor- κβ
NIPC: Natural interferon producing cell
NK cell: Natural killer cell
ODN: Oligodeoxynucelotide
Opn-i: Internal Osteopontin
OVA: Ovalbumin
PALS: Periarteriolar lymphoid sheath
PAMP: Pathogen associated molecular pattern
PBMC: Peripheral blood mononuclear cell
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
pDC: Plasmacytoid dendritic cell
PDCA-1: Plasmacytoid dendritic cell antigen 1
Pen/Strep: Penicillin/Streptomycin
PFU: Plaque forming units
PI: Propidium iodide
PIR: Paired Ig-like receptor

PLN: Popliteal lymph nod
PRR: Pathogen recognition receptor
RAG: Recombination activating gene
RAGE: Receptor for advanced glycation end product
RIG-I: Retinoic acid-inducible gene I
RSV: Respiratory syncytial virus
SA: Strepavidin
SHIP: Src homology 2 domain-containing inositol-5-phosphatase
SHP-1/-2: Src homology- phosphtases -1/-2
Siglec: Sialic acid binding Ig-like lectins
SLE: Systemic lupus erythematosus
ssRNA: Single stranded RNA
TAP: Transporter association with peptide processing
TCR: T cell receptor
Tg: Transgenic
TH1: T helper 1
TH2: T helper 2
TIR: Toll/IL-1 receptor domain
TIRAP/MAL: TIR-domain containing adaptor protein
TLR: Toll-like receptor
TNF-α: Tumour necrosis factor-α
TRAF: Tumor necrosis factor receptor associated factors
TRAM: TRIF-related adaptor molecule
TRIF: TIRAP inducing IFN-α
VEGF: Vascular endothelial growth factor
Vpu: Viral protein u
VSV: Vesicular stomatitis virus
WT: Wildtype

CHAPTER 1. INTRODUCTION (Review of the literature)

1. Plasmacytoid dendritic cells

1.1. History

The field of plasmacytoid dendritic cell research originated more than half a century ago and was initiated by Isaacs and Lindenmann investigating the concept of "viral interference" (1). This phenomenon describes the ability of virally infected cells to block subsequent cellular infection by a second virus when grown in the same culture. Isaacs and Lindenmann discovered that this "interference" was mediated by a soluble factor that was transferred by the infected cells to uninfected cells and conferred viral resistance on the cell culture. They termed this substance "the interferon" and set the stage for the development of the fields of interferon and dendritic cell biology (1).

In subsequent studies of virus interactions with mononuclear cells in the peripheral blood, type I interferon (IFN) was discovered to be rapidly produced and released by these cells into the culture supernatant (2). Monocytes were initially presumed to be responsible for the production of type I IFN (3). However, it was subsequently found that the blood cell responsible for the production of the majority of type I IFN was present in low frequency, major histocompatibility complex (MHC) class II positive, and distinct from T cells, B cells, monocytes and Natural Killer (NK) cells (4-6). Many cell types are capable of producing type I IFN in response to viral stimulus. However, these lineage-negative cells were found to produce up to 3-10pg/cell of IFN-α in response to viral stimulus, representing an amount that is 10-100 times more than any other

cell type. Alm's group (7) called these cells Natural interferon producing cells (NIPC) and categorized them as belonging to the innate branch of the immune system then known as natural immunity. Evidence from both Rinaldo (8) and Fitzgerald-Bocarsly's (9) group indicated that these NIPC may be related to dendritic cells (DC) although they were clearly distinct from myeloid DC (mDC). In 1958, the pathologists Lennert and Remmele (10) described cells with plasma cell-like morphology located in T cell zones of human spleen and lymph nodes. In the decades to follow, these cells were erroneously described as "plasmacytoid T cells" or "plasmacytoid monocytes" based on their lymphoid tissue localization and cell surface markers.

The identification of NIPC as a subset of DC came from Liu's lab (11) in 1997 when these cells were isolated from T cell-rich areas near high endothelial venules (HEV) in human tonsils. They were termed DC2 based on their ability to prime T helper 2 (TH2) responses when cultured with IL-3 and CD40 ligand. Under electron microscopy these DC2 displayed abundant cytoplasm with extensive endoplasmic reticulum. The term "plasmacytoid" comes from this morphological comparison of DC2 with mature, antibody-secreting plasma cells. However, the protein that these cells were poised to produce was not identified.

The fields of interferon research, pathology and dendritic cell biology converged when it was determined that the NIPC, which made large amounts of IFN-α in response to viral and synthetic stimuli, were identical to the plasmacytoid subset of DCs – the Plasmacytoid dendritic cells (pDCs) (12). The first reports of murine pDCs, which share most of the human morphological and

functional characteristics, appeared in 2001 (13-15). The discovery of the mouse pDC population greatly aids in dissecting the role of this important DC subset in humans. Presently, the field of pDC research has reached new heights. Recent studies of both human and mouse pDCs reveal important roles of this unique and important cell type in viral infection, autoimmunity and other human diseases.

1.2. Origin and development

The majority of DC research has been carried out using myeloid dendritic cells (mDCs). In humans, monocyte-derived DCs (MDDCs) represent the most commonly used model system for studying mDCs. Peripheral blood monocytes are cultured in the presence of granulocyte macrophage - colony growth stem cell factor (GM-CSF) and IL-4. On day seven, MDDCs are non-adherent cells that lack the cell surface marker CD14, display DC morphology and are highly efficient at capturing antigen. In mice, mDCs are generated by culturing bone marrow cells in the presence of GM-CSF or Fms-like tyrosine kinase 3 – ligand (Flt3L) (16). Flt3L is also the only known growth factor that allows for the differentiation of human and mouse pDCs. Flt3L cultured DCs must be further separated into mDC and pDC populations by cell sorting. Administration of Flt3L in vivo to humans and mice results in the migration of both sets of DCs into the periphery (17-20).

While human and mouse mDCs can be clearly derived from a common myeloid precursor in bone marrow, the lineage origin of pDCs remains controversial. Studies favoring the myeloid lineage of pDCs points to the pDC expression of the GM-CSF receptor and interferon regulatory factor -8 (IRF-8) (a myeloid transcription factor), present only on cells derived from the myeloid

lineage (21). Later studies propose a lymphoid lineage based on pDC expression of the pre-T cell receptor-α, a partially rearranged Ig heavy chain (IgH), V-pre B, Lambda5, Spi-B and notch-1, which are all gene products associated with the lymphoid lineage (22). Further evidence for the lymphoid origin of pDCs comes from the developmental inhibition of human T cells, B cells and pDCs that ectopically express the transcription factors Id2 and Id3 (23). Presently, it is believed that there is considerable plasticity to DC lineages. Shigematsu et al. (24) demonstrated that pDCs could be derived from either myeloid or lymphoid precursor cells and that lymphoid associated transcripts (RAG, IgH) can be found in both myeloid and lymphoid-derived pDCs. Furthermore, Oldstone's group recently demonstrated the actual conversion of mouse bone marrow (BM)-derived naïve pDCs into mDCs upon infection with lymphocytic choriomeningitis virus (LCMV). They demonstrated that the conversion was independent of cell division of the naïve pDCs, involved production of type I IFN and occurred only upon infection of immature BM pDC and not of peripheral pDCs. They further suggest that this reversible programming of pDCs into mDCs may efficiently sustain adaptive immune responses (25).

Various transcription factors have recently been shown to influence pDC development and function. Mice lacking IRF-4 and 8 lacked both pDCs and mDCs. IRF-8, and to a lesser extent, IRF-4 was found to be essential for pDC development (26). The zinc finger protein Ikaros is another transcription factor that has been found to be strongly associated with pDC development. Mice containing low levels of Ikaros expression lacked peripheral blood pDCs and were

subsequently unable to produce IFN-α in response to Toll-like receptor (TLR)-7 and TLR-9 agonists (27, 28).

1.3. Morphology and surface phenotype

Plasmacytoid DCs are said to be plasmacytoid because of their plasma cell-like morphology under Giemsa staining. They are round cells with a kidney-shaped eccentric nucleus. They are larger than resting lymphocytes, but smaller than monocytes. By electron microscopy, pDC, like plasma cells, have a well-developed endoplasmic reticulum, which is characteristic of secretory cells (29). Only resting pDCs have the plasmacytoid morphology. Upon activation, the membrane protrudes to form dendrites that are characteristics of mDCs (11). Therefore, the term "plasmacytoid precursor dendritic cell" is sometimes used in the literature since it more accurately describes the real nature of pDCs (14).

Mouse pDCs are characterized by the following cell surface markers: $CD11c^{low}$; B220 (CD45); Ly6C (recognized by anti-Gr1 mAb to Ly6G, but cross reacts with Ly6C); low expression of MHC class II and costimulatory molecules on resting pDC (however, both MHC class II and costimulatory molecules are elevated in activated pDC) (14); CD4 and CD8 expression is variable and linked with developmental status (14, 30); bone marrow stromal cell antigen-2 (BST-2/Tetherin/CD317) (recognized by 120G8 and mPDCA-1, but is expressed on resting B cells and other cell types upon IFN-α and IFN-γ treatment) (31, 32); sialic acid binding Ig-like lectin-H (Siglec-H) (which is recognized by mAb 440c) (33); pDC-Triggering receptor expressed on myeloid cells (pDC-TREM) (34);

and Ly49Q, a lectin-like class I MHC receptor belonging to a family of receptors expressed mainly on NK cells (which is recognized by 2E6 and NS34) (35).

1.4. Differentiation from mouse myeloid DCs

Dendritic cells are often referred to as the sentinels of the immune system. This is because they play a pivotal role in the initiation of the adaptive response by up-taking, processing and presenting antigenic material to T lymphocytes in the primary and secondary lymphoid organs. DCs are a heterogeneous population of bone marrow-derived cells sharing and differing in morphological and functional characteristics (36, 37). They link innate resistance mechanisms with the adaptive immune response by interacting with various cell types. They also help to establish microenvironments favorable to T cell priming through the secretion of pro-inflammatory cytokines. In addition to their crucial role in activating the immune response, DCs also participate in peripheral tolerance (38, 39). These diverse roles suggest that the DC population consists of a variety of subsets that are capable of exerting specific functions.

A myriad of DC subsets have been characterized in humans and mice. Initial mouse DC classifications that relied on T cell markers (CD4 and CD8) that had no known functional role on the DCs are no longer used (40). The CD8 marker is absent on human DC, which confounded a parallel classification in mice and human DCs (41).

The current DC classification scheme accepts that all mice DCs are $CD11c^+$ with variable expression of CD4, CD8 and low to high expression levels of costimulatory molecules and MHC class II. Based on immune functions, the general acceptance is two subsets of DCs: mDCs and pDCs. Myeloid DCs are

defined as CD11c^{high}, CD11b^{+} and MHC class II^{+} and are the most commonly referred to in the literature (42). The term "myeloid" comes from the generally accepted developmental origins of mDCs, which are thought to have descended from myeloid progenitor cells. Maximal surface area and sampling of environmental antigens is achieved through long extensions or "dendrites" that protrude from the cell body of the mDC. A distinct set of cell surface receptors are expressed on mDCs include pathogen recognition receptors and internalization receptors. Myeloid DCs express TLRs -2, -3, -4, -6 and -7. CD80/86 maturation molecules are up regulated, while higher expression levels of constitutive MHC class II are expressed once danger signals are sensed by the mDC. Pathogen internalization is stopped, antigenic material is processed and the activated mDC seeded in its organ/tissue site is recruited to the draining lymph node through afferent lymph for antigenic presentation. Once homed to the lymph nodes, high expression of MHC class II, costimulatory molecules and the secretion of cytokines (mainly IL-12, IL-6, Tumour necrosis factor (TNF)-α and low amounts of IFN-α) allow for efficient T cell priming and initiation of adaptive immunity. It is these characteristics that define mDCs as the most potent Antigen presenting cell (APC).

In contrast to mouse mDCs, mouse pDCs have poorly defined developmental origins, constitutively circulate in blood, are thought to migrate to lymph nodes through HEV, express the endosomal TLRs -7 and -9, and display relatively poor costimulatory and T cell priming abilities (43-45).

1.5. Differentiation from human pDCs

In contrast to mouse pDCs, human pDCs are negative for all lineage markers including CD11c (43). Human pDCs can be distinguished from other blood cells based on the selective expression of blood dendritic cell antigen -2 (BDCA-2) and ILT7 (45-47). Human pDCs also express BDCA-4/neuropilin (48) and Nkp44 (49), DCIR (50), BST-2 (31, 47), FcγRIIa (51), IL-3Rα (CD123), CD4, MHC class II and CD2 (43). CD2 high expressing pDCs have been found to express lysozyme, but pDC cytolytic capacity is questionable. Furthermore, human pDCs express two intracellular proteins, granzyme B and the CD2-associated protein – CD2AP (52). Culturing BM precursors with IL-3 and Flt3-L efficiently induces pDC differentiation. In addition, human pDCs do not produce the cytokine IL-12 (43, 53), while mouse pDCs can secrete this cytokine given the appropriate antigenic stimulant (43). Toll-like receptors -7 and -9 are also expressed on human pDCs along with the various signaling components leading to type I IFN secretion (54). Further comparisons between human and mouse pDCs will be address in detail in each subsequent section of this thesis.

Human pDC studies are limited to direct isolation and *in vitro* culture of pDCs from blood while mouse studies are facilitated by the availability of different lymphoid organs. The major differences between human and mouse pDC subsets are thought to be accounted for in part by these technical limitations.

1.6. Frequency and distribution

Functional studies of pDCs are inherently difficult due to their very low frequency in both human and mouse peripheral blood and lymphoid tissue.

Plasmacytoid DCs represent approximately 1% of leukocytes in the bone marrow and spleen and less than 0.5% in the lymph nodes and peripheral tissues (32). In mice, the frequency of pDC varies between strains; with 129Sv mice have significantly higher proportion of pDCs than any other strain. For example, 5 times more splenic pDCs were isolated from 129Sv mice compared to C57BL/6 mice (B6) (32). Plasmacytoid DCs survive for approximately 2 weeks *in vivo* and constantly emigrate from the bone marrow and colonize both lymphoid and non-lymphoid organs in small numbers (30). *In vitro*, freshly isolated pDCs are extremely culture labile and require TLR agonists for survival beyond a few hours (45)

1.7. Migration pattern and localization

Similar to naïve T cells and B cells, pDCs constitutively circulate in blood and migrate to lymph node through HEVs (11, 32, 55, 56). CD62L (L-selectin) expression by pDC mediates extravasation through the HEV (11, 56). The absence of CD62L results in reduced pDC numbers in lymphoid organs (13). This process is also facilitated by the expression of a wide panel of chemokine receptors. In particular, the chemokine CCR7, which binds chemokines CCL19 and CCL21, secreted by stromal cells within the T cell area, is important for pDC migration (56). Other pDC expressed chemokine receptors involved in pDC trafficking to secondary lymphoid organs include CXCR3, CCR1, CCR2, and CCR5 (56, 57).

In the resting state, pDCs localize in the inner T cell rich areas of both spleen and lymph nodes and in the marginal zone and red pulp of spleen. They tend to accumulate around the HEV of LN (32, 55). Under inflammatory

conditions, pDC accumulate as clusters in lymphoid organs (14). Studies have demonstrated that pDCs migrate constitutively to LN and migration is further enhanced after pDC activation (55, 56, 58)

Recent studies, however, shed new light on whether pDC entry into LNs proceed solely through HEV. *Pascale et al*. (59) describe pDCs in the afferent lymph of noninflamed skin of sheep and pigs in a similar proportion relative to mDCs to that observed in the blood or lymphoid organs. Plasmacytoid DCs are difficult to detect in most peripheral tissue of mice and rats including afferent lymphatics and therefore have been thought to exclusively migrate to LNs through HEV. Species intrinsic differences in pDC migration, differences in the amounts of pathogen exposure in the animals used in each study and the retainment of pDC in the LNs after recruitment have been proposed to address this apparent discrepancy (59).

1.8. Viral recognition by pDCs

1.8.1. Toll-like receptors

To detect the presence of infection, the innate immune system first recognizes conserved molecular structures that are predominantly found in microorganisms, but not in vertebrates (60). Pathogen associated molecular patterns (PAMPs) are recognized by various types of pattern recognition receptors (PRRs) expressed on immune cells including monocytes, macrophages, DCs, B cells and NK cells (61). TLRs are a family of conserved membrane-spanning molecules that contain an ectodomain of leucine-rich repeats, a transmembrane domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain (60). Since their initial discovery in *Drosophila melanogaster*, ten different human TLRs and 12 in

mice have been described (62-64). The extracellular leucine-rich repeats of the TLR bind to PAMPs such as lipopolysaccharide (LPS) of bacterial cell wall (TLR-4 ligand) (65), bacterial flagellin (TLR-5 ligand) (66), and bacterial lipoprotein and peptidoglycan (ligand recognized by the complex of TLR-2 with TLR-1 and TLR-2 with TLR-6) (67). Unlike these TLRs, which are expressed on the host surface to mediate detection of microorganisms in the extracellular environment, TLR-3, TLR-7, TLR-8 and TLR-9 (68-71) are located in intracellular endosomal compartments where they recognize microbial nucleic acids.

Human and mouse pDCs selectively express endosomal TLR-7, TLR-8 (pseudogene in mice) that recognize single stranded viral RNA and TLR-9 that recognizes CpG DNA found in microbial genomes (71, 72). Ligand binding leads to TLR activation and the recruitment of cellular adaptor molecules that contain TIR domains and the formation of signal transduction complexes in the cytoplasm (61, 73). In various combinations, the adaptor proteins myeloid differentiation primary-response gene 88 (MyD88), TIR-domain containing adaptor protein (TIRAP/MAL), TIRAP inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM) directly bind to TLRs (74-76). With respect to the pDC-specific TLRs, only MyD88 has been shown to play a major role of adaptor protein upon TLR-7 and TLR-9 engagement (77, 78). TLR-3 and TLR-4 signal through TRIF (75, 79, 80) and TLR-4 can also signal through TRAM (81). Multiple signaling pathways including nuclear factor-κβ (NF-κβ), Mitogen-activated protein kinases (MAPKs) and IRFs are activated downstream of TLR-

adaptor molecule binding. This ultimately leads to the transcription of genes encoding cytokines, chemokines and co-stimulatory molecules (60, 75, 82-84).

1.8.2. Non toll-like receptors expressed on pDCs

As professional IFN-α producing cells, pDCs must express receptors that are able to internalize a wide variety of viruses. Recent esearch progress has been made on understanding concerning the mechanisms that mediate the delivery of viral components to endosomal TLR-7 and TLR-9 and on the characterization of how these receptors modulate TLR-induced IFN-α production.

TLR ligand delivery via endosomes and authophagy has been characterized. Viruses like vesicular stomatitis virus (VSV) and Sendai virus (Sev) enter pDC through direct fusion and then generate replicative intermediates that are redirected from the cytosol into TLR-7 and TLR-9 containing endosomes (45). Through the process of autophagy, vacuole formation is generated by encirclement of the viral intermediate followed by direct fusion with TLR-7/-9 containing endosomes (85). Other viruses, like herpes simplex virus (HSV) and Coxsackievirus B (CVB) are internalized via receptor-mediated endocytosis and delivered directly to TLR-7/-9 endosomes (86, 87). It has been suggested that the delivery of viral nucleic acids into intracellular TLR-7/-9 containing endosomes requires a tight spatiotemporal regulation of membrane trafficking involving pDC receptors that modulate IFN production (88).

Plasmacytoid DCs express various cell receptors that modulate the amplitude of type I IFN responses to TLR-7 and TLR-9 ligands (45). These receptors can be categorized into two major groups. The first group of receptors

requires the presence of an adaptor molecule DNAX activation protein-12 (DAP-12) or the γ chain of Fc receptors (FcRγ). The Immunoreceptor tyrosine-based activation motif (ITAM) that is contained intracellularly within these adaptors becomes activated by phosphorylation upon receptor association and recruits the protein tyrosine kinase Syk. The second group of receptors contains cytoplasmic Immunoreceptor tyrosine-based inhibitory motifs (ITIM) that are phosphorylated upon receptor engagement and recruit protein tyrosine phosphatases (SHP-1/-2) and/or phosphatidyl inositol phosphatase (SHIP-1/-2). Although receptors that signal through ITAM usually activate cells and ITIM-bearing receptors inhibit cell activation, this rule does not always hold true in both mouse and human pDCs (45).

Human pDCs express BDCA-2, a C-type lectin receptor, which associates with the signaling adaptor FcRγ chain. While the natural ligands for BDCA-2 are unknown, antibody directed to this receptor was subsequently internalized into endosomes, processed and presented to T cells (89). Furthermore, cross-linking of BDCA-2 has been shown to inhibit the ability of pDCs to produce IFN (89, 90). Recently, antibody mediated triggering of BDCA-2 was demonstrated to result in tyrosine phosphorylation of Syk, Lyn, Btk, BLNK, and other components of the B cell receptor signaling complex (91).

The Immunoglobulin-like transcripts receptor ILT7 was shown to associate with the signal adaptor protein FcεRIγ (46) and presumed to activate human pDCs through immunoreceptor tyrosine-based activating motif (ITAM) mediated signaling. However, ILT7 ligation with Cytosine-phosphate-Guanine

oligodeoxynucleotides (CpG-ODN) or Influenza virus inhibited pDC production of type I IFN and other cytokines (46). Recent progress studying the ILT7 human pDC receptor reveals the identification of its ligand bone marrow stromal antigen 2 (BST2), which initiates signaling via the ILT7- FcεRIγ complex, and strongly inhibits production of IFN-α and pro-inflammatory cytokines by pDCs (47). It is interesting to note both ILT7 and BST2 are expressed on pDCs.

The NK cell-mediated cytotoxicity receptor NKp44 is found on human pDCs in the tonsils (49) . This receptor is normally expressed on NK cells, associates with DAP-12 and initiates cytotoxicity (92, 93). In NK receptors, DAP-12 contains an ITAM motif and downstream signaling involves activation of cellular effector events (94). NKp44 is also induced on human blood pDCs after in vitro culture with IL-3 (49). It has been found that cross-linking of NKp44 in combination with CpG-ODN inhibits IFN-α production by blood pDCs (49). Colonna's group speculates that IL-3 secreted by memory $CD8^+$ T cells induces NKp44 expression on blood pDCs and controls pDC anti-viral responses (49). In contrast to NK containing DAP-12, DAP-12 associated pDC receptors appear to produce inhibition of cellular activation (45).

Another DAP-12 associated family of receptors, the Siglecs/CD33 has been studied on both human and mouse pDC. The Siglecs structurally belong to the immunoglobulin superfamily and traditionally binds to sialic acids present on a variety of cell surface glycoproteins (95). Siglec 5 is expressed on human pDCs, recognizes *Neisseria* species and mediates its phagocytosis (96, 97). Siglec-H is recognized by the 440c monoclonal antibody and is expressed

predominantly on mouse pDCs. Other studies have shown expression patterns of Siglec-H on mouse macrophage subsets and brain cells (97). Recently, Zhang et al. demonstrated that Siglec-H can function as an endocytic receptor, mediating antigen uptake and presentation to T cells (98). Furthermore, 440c mediated cross-linking of Siglec-H on *ex vivo* pDCs resulted in inhibition of IFN-α secretion in response to TLR-9 stimulation both *in vitro* and *in vivo*. In confirmation, DAP-12 deficient pDCs (with unstable Siglec-H cell surface expression) produced more IFN-α following CpG stimulation (33).

The Dendritic cell immunoreceptor (DCIR) is an ITIM-containing receptor expressed by human pDCs (50). Cross-linking of DCIR inhibits the production of TLR-9-induced IFN-α, but has no effects on TLR-9 induced pDC maturation. Furthermore, DCIR may function as an antigen capturing molecule as antigens targeted to pDC via DCIR are presented to T cells (50).

Functionally similar to BDCA-2, human pDCs express BDCA-4, which is also known as the neuronal receptor neuropilin-1. BDCA-4 is a co-receptor to a tyrosine kinase receptor for both vascular endothelial growth factor (VEGF) and semaphorine. Prior to its discovery on human pDCs, BDCA-4 was known to play versatile roles in angiogenesis, tumor invasion, cell survival and axon guidance (99). In the context of pDCs, anti-BDCA-4 mAb cross-linking and CpG-ODN stimulation has resulted in reduced IFN-α secretion (48). Anti-BDCA-4 mAb cross-linking on human pDCs also appears to affect cell survival and block viral attachment (48) .

In addition to C-type lectin receptors, human pDCs have additional antigen capturing receptors. FcγRIIa and FcεRI can endocytose intact viruses, antibody opsonized viruses or immune complexes containing DNA or RNA. This results in the stimulation of IFN-α production through TLR-7 and -9 (51). It was shown by Finberg's group that antibody opsonization of the ssRNA picornavirus, Coxsackievirus B, lead to virus uptake by mouse pDCs through FcγRII and induction of IFN-α via TLR-7 (87, 100).

PDC-TREM is a member of the Ig-superfamily and associates with the adaptor DAP-12. It is exclusively expressed by activated mouse pDCs. In contrast to Siglec-H, PDC-TREM enhances IFN-α secretion enduced by TLR-9 signaling (34).

Mouse pDCs also express BST-2, a type II transmembrane glycoprotein containing a modified glycosylphosphatidyl-inosinol luminal (GPI) – linked domain (101). BST-2 has been shown by Colonna's group (31) to be recognized by the pDC specific mAbs, 120G8 and mPDCA-1. However, BST-2 expression also appears on other activated immune cell types such as T cells and B cells (31). Recently, it has been demonstrated that the human immunodeficiency virus (HIV) accessory protein viral protein u (Vpu) down regulates BST-2 expression on infected cells (102), highlighting once again the intricate interplay between viral proteins and host cellular factors. In pDCs, it is conceivable that BST-2 is involved in sorting important secreted proteins, notably IFN-α, as BST-2 expression has also been observed in intracellular compartments (31).

Lastly, the human pDC Receptor for advanced glycation end products (RAGE) has been shown to recognize DNA/High-mobility group box 1 (HMGB1) (nuclear DNA bind protein released by necrotic cells and CpG stimulated pDC) complexes and result in IFN-α production by pDC. HMGB1 appears to bind CpG-A and results in increased IFN-α production through both TLR-9 and RAGE [72]. However, other studies have shown reduced IFN-α production by HMGB1 [73]. Further studies are necessary to assess the roles and underlying mechanisms of all these receptors in pDC cytokine secretion.

1.8.3. IFN-α production and other secreted products

Type I IFN secretion is presently the most important function of pDCs in immune responses. Plasmacytoid DCs have been designated as the "professional" IFN producing cells because they are capable of producing 10-100 times more IFN-α than any other cell type (43). The magnitude of IFN responses in pDCs is illustrated by the observation that as much as 3-10pg/cell of IFN-α is produced in response to HSV-1 stimulation (103).

This remarkable ability to produce IFN-α is due to several factors. One key difference between pDCs and other cell types is that TLR-7/-9 ligation induces efficient production of type I IFNs in pDCs, but not in other cell types expressing them, like B cells. TLR-7 recognizes single stranded RNA viruses as well as synthetic agonists including compounds in the imidazaquinoline family (104, 105). TLR-9 binds double stranded microbial DNA and more specifically to CpG ODN. These are DNA motifs rich in cytosine and guanine. CpG motifs in vertebrates are rare and are methylated specifically on cytosine (106). The

unmethylated CpG motifs of bacteria and the very high content of some viral genomes for CpG islands can trigger the TLR-9 pathway. Synthetic CpG preparations with specific sequences and free of endotoxins are commercially available. These preparations have been shown to differentially modulate pDC maturation and secretion of IFN-α depending on the sequence of the CpG ODN (107).

There are two classes of CpG-containing DNA sequences and they have been reported to elicit differential cytokine responses in pDCs. Type A CpG ODNs are characterized by a polyG tail that mediates the spontaneous formation of large multimeric aggregates. It is thought that this multimeric structure prolongs the retention time of CpG-A in the early endosomes and therefore provides the opportunity for extended activation of the signal-transducing complex, MyD88 and IRF-7 (82). This prolonged TLR-9 signaling from early endosomes is thought to lead to robust IFN-α production by pDCs. In contrast, CpG-B ODNs are monomeric, do not form multimeric structures and thus they cycle quickly through early endosomes into late endosomal-lysosomal compartments. This endosomal difference presumably activates a different set of signal mediators downstream of MyD88/TRAF6/IRAK4, including Nuclear factor k B (NFkb) and mitogen-activated protein kinases (MAPKs) and IRF-5 (78). This has been shown to induce the production of the pro-inflammatory cytokines TNF-α and IL-6, small amounts of IFN-α and the up-regulation of CD80, CD86 and expression of MHC class II molecules (108-110). Conversely, liposome

encapsidation of CpG-B ODNs allows for their retention in early endosomes and the subsequent production of much higher levels of IFN-α (32).

However, in our hands, low levels of CpG-B ODN in the absence of lipid complexing do induce IFN-α production by *ex vivo* splenic pDCs (unpublished observations). Studies by other groups using freshly isolated splenic, thymic and bone marrow pDCs (30, 111) also demonstrate IFN-α secretion in response to CpG-B ODN. This appears to be at odds with the retention argument and suggests the existence of other cellular mechanisms that modulate pDC IFN-α secretion.

In addition to producing copious amounts of IFN-α in response to viral stimulation, pDCs also produce IFN-β as well as IFN-κ and IFN-ω in response to viral stimulation, but at much lower levels (103, 112). Little is known about the subtypes of IFN-α produced by pDCs in the course of a viral infection due to the lack of antibodies that can distinguish between the 14 subtypes of IFN-α in existence (113). In addition to type I IFNs, pDCs also produce type III IFNs known as IFN-λ in response to viral stimuli or TLR-7 and TLR-9 agonists (104). The IFN-λ receptor has limited cellular expression in contrast to the IFNR, which is expressed on many cell types (104).

In addition to IFNs, TLR-activated pDCs produce high levels of TNF-α and IL-6. The maturation of pDCs into antigen-presenting cells is in part driven by TNF-α (105, 114). IL-6 along with IFN-α has been found to induce the differentiation of antibody secreting plasma cells (115, 116). Human pDCs do not

produce significant amounts of IL-12p70, the Th1-inducing cytokine, whereas mouse pDCs do in response to TLR ligands (15).

Plasmacytoid DCs also produce chemokines in response to viral stimuli. These include CCL2, CCL3, CCL5, CXCL10 and IL-8 (55, 117, 118). These chemokines are able to recruit activated T cells and NK cells (117). In terms of chemokine receptors, pDCs express CXCR3, CXCR4 and CCR5, which allow pDCs to migrate in response to other chemokines (119).

In addition to cytokines and chemokines, pDCs also express the anti-microbial peptide human beta-defensin 1 (HBD-1) in response to virus stimulation (120, 121). Little is known of the significance of HBD-1 in pDC biology, but studies by Fitzgerald-Boscarly's group indicate anti-HIV-1 properties of HBD-1 (121).

1.8.4. Intracellular signaling

IFN-α production by pDCs following viral stimuli proceeds via MyD88-dependent and independent pathways (122, 123). The binding of CpG DNA or viral ssRNA to endosome anchored TLR-7 or TLR-9 triggers the recruitment of the MyD88 adaptor molecule (82). MyD88 complexes with the IL-1 receptor associated kinase (IRAK) family, IRAK-1 and IRAK-4. This forms the Cytoplasmic transductional-transcriptional processor (CTTP) (124). Accordingly, Delale et al. show that both TLR-9- and MyD88-deficient mice were susceptible to MCMV infection. However, MyD88-deficient mice showed a more defective IFN-α response (123). The more severe phenotype in MyD88 deletion makes sense given that multiple TLRs converge on this adaptor molecule (78). The

activated IRAKs -1 and -4 further associate with IRF-5 and -7, the tumor necrosis factor receptor associated factors (TRAF)-6 and –3, and the Ikβ kinase (IKKα) (16). After being phosphorylated by IRAK-1 and activated by TRAF-6, IRF-7 translocates into the nuclei to initiate type I IFN transcription (82, 83). IRF-7, thus, serves as a master regulator of IFN responses (82, 83, 124) (Figure 1).

Unlike other peripheral blood mononuclear cells (PMBCs), pDCs have been found to constitutively express high levels that IRF-7 that can quickly translocate to the nucleus following virus stimulation (83, 124). Work by Trinchieri's group revealed that the IRF-7 signaling pathway proceeds in the absence of IFN feedback signaling in virus-infected mice, but not in response to CpG (58). Research conducted by Taniguchi's lab demonstrated that IFN-α production was intact in IRF-3 deficient mice, but not in IRF-7 deficient mice (124). In addition to IRF-7, both human and mouse pDCs strongly express IRF-4, -5 and -8 (26, 112). Re-introduction of IRF-8, but not IRF-4, back into IRF-4/IRF-8 double knockout mice restored the ability to produce IFN-α. This suggests an important role for IRF-8 in pDC IFN-α production (26). Investigations into the roles of IRF-4 and -5 in IFN-α production by pDCs have yielded contrasting results by various groups and remains under investigation (26, 112, 125).

In addition to the aforementioned components of the CTTP, internal Osteopontin (Opn-i), a virally induced phosphoprotein that is expressed in DCs, macrophages and T cells, has been demonstrated to play an important role in pDC IFN-α secretion. Using $Opn^{-/-}$ mice, Shinohara et al. demonstrated a deficiency in

TLR-9-dependent IFN-α production, but not in NFkβ-dependent pro-inflammatory cytokines (126). Opn-i was found to co-localize with MyD88 and lack of Opn-i resulted in deficient IRF-7 translocation in pDCs. In addition, Opn-$i^{-/-}$ mice demonstrated deficient *in vivo* IFN-α response to Herpes simplex virus (HSV) challenge, deficient IFN-activated NK activity and deficient IFN-dependent cross-presentation of antigen by pDCs (126, 127).

Plasmacytoid DCs also express two viral RNA sensors that reside in the cytosol, Retinoic acid-inducible gene I (RIG-I) and Melanoma differentiation-associated gene 5 (MDA5). RIG-I has been shown to preferentially recognize ssRNA that is phosphorylated at the 5` terminus, short dsRNA molecules and certain RNA viruses (128, 129). MDA-5 recognizes long dsRNA, polyinosinic-polycitidylic acid (PolyI:C) and picornaviruses (129, 130). Recognition of viral nucleic acid by RIG-I and MDA-5 takes places after viral entry directly into the cytoplasm and recruits the adaptor IFN-β promoter stimulator-1 (IPS-1) (131, 132). This results in activation of IRF-3 and IRF-7 to induce the transcription of type I IFN and antiviral genes (85). This TLR independent pathway that leads to IFN-α production exists in other immune cells (mDCs) as well as non-hematopoietic cells (fibroblasts), but its specific role in pDC IFN-α production is controversial (Figure 1). A survey of the literature reveals pDC-independence and –dependence on cytosolic RNA receptors. Using RIG-I-deficient mice, Kato et al. demonstrate that RIG-I is essential for induction of type I IFN after infection with RNA viruses in fibroblasts and mDCs, but not in RIG-deficient pDCs, which produced large amounts of IFN-α (130, 133, 134). In parallel studies, Sun et al.

demonstrated abolished viral induction of type I interferon using IPS-1-deficient mice in mDCs, macrophages and fibroblasts, but not in pDCs (130). In contrast to the evidence provided for the dispensability of cytosolic receptors in pDC, Hochrein et al. describes a pDC TLR-9 independent induction of IFN-α in response to HSV that appeared to be heat sensitive, suggesting a role for an RNA intermediate in the signaling pathway (86). A recent study conducted by Kumagai et al. tries to reconcile these opposing data. Using IFN-α-GFP mice that allowed the *in vivo* monitoring of type I IFN production, they demonstrate RIG-I-dependence of pDC IFN-α production in the absence of IFNR signaling. They show that the TLR system along with type I IFN feedback signaling in pDC circumvents the requirement for viral replication and RIG-I activation. However, the lack of type I IFN feedback or TLR permits viral replication in pDCs and subsequent RIG-I/IPS-1 activation. In addition to cytoplasmic RNA sensors, no evidence yet exists for the presence of cytoplasmic DNA sensors in pDC IFN-α production (29, 135).

1.9. Interaction with other cell types

The extraordinary ability of pDCs to produce IFN-α in response to microbial invasion and its localization in T cell zones of peripheral lymphoid organs uniquely places pDCs at the interface between innate and adaptive immune responses.

1.9.1. Innate immune cells

Cross-talk between pDCs and various cell types is likely due to the central role of type I IFN in many immune responses. IFN-α directly acts on mDCs as

mDCs do not mature during mouse cytomegalovirus (MCMV) infection in the absence of type I IFN receptor (136). IFN-α also enhances the production of various cytokines. For example, IFN-α appears to inhibit or enhance the expression of the different IL-12 subunits under certain conditions. It has been reported that in the absence of IFN-α, there is a dramatic increase in IL-12 secretion during MCMV infection (137). Myeloid DCs and pDCs can also interact in a cell-contact dependant manner. Tam and colleagues demonstrated that mouse mDCs infected with *L. monocytogenes* secrete IL-12 and IL-15, and that the IL-15 originates from CD40 and CD40L interactions between mDCs and pDCs, respectively (117). Furthermore, IL-15 sustains IL-12 production (138). Since IL-12 is pivotal in the outcome of T cell responses, interaction between pDCs and mDCs requires greater investigation. IFN-α also enhances the maturation of human $CD11c^+$ mDCs, with mDC matured by IFN-α leading to induction of IL-10-producing T regulatory cells (139).

Another important interaction involves crosstalk between pDCs and NK cells. Both mDCs and pDCs have been demonstrated to contribute to virus-induced activation of NK cells through type I IFN-dependent mechanisms (140). Briere's group demonstrated that pDCs can improve NK cell responses to MCMV infection. Plasmacytoid DCs secrete IFN-α after recognition of dsDNA of MCMV through TLR-9 and it is this IFN-α that primes NK cell responses. Depletion of pDCs impairs NK responses to MCMV infection (105). Furthermore, TLR-9-, MyD88- and IRF-7 deficient pDCs are increasingly impaired to prime NK cell defenses against MCMV. However in the absence of

pDC (or impaired pDC function), there is increased IL-12 secretion *in vivo* by mDCs and other innate cells that aid NK cells in combating MCMV infection (123). In addition to the NK activating effects of pDCs, pDCs also participate in NK homing by producing chemokines that selectively recruit both NK cells and activated T cells (117). *In vivo*, this could occur either in the lymph node or at a site of infection, resulting in the recruitment of vital anti-viral effector cells.

1.9.2. Adaptive immune cells

Resting pDCs are very poor inducers of T cell responses (11). They express low levels of MHC class II and co-stimulatory molecules. Once activated by TLR-7/-9 ligands, pDCs increase their cell surface expression of co-stimulatory molecules as well as MHC Class I and Class II molecules (12, 14). Upon maturation, pDCs lose their capacity to produce IFN-α and gain the capacity to present antigen.

Plasmacytoid DCs have been shown to present both endogenous and exogenous antigen. Several studies have shown that pDCs present endogenous antigens via MHC I and II molecules, both constitutively expressed and virus derived antigens (141). The process of autophagy allows pDCs to sense viral nucleic acid intermediates and deliver them to TLR-7 and -9 containing endosomes (85). It has been speculated by Paludan et al. that this is likely the same mechanism employed by pDCs to deliver cytosolic antigens to MHC II containing endosomes (142). The mechanisms governing exogenous antigen uptake in pDCs are speculative at best. Plasmacytoid DCs have been shown to efficiently capture ovalbumin both *in vitro* and *in vivo* by macropinocytosis or

receptor-mediated endocytosis. Potential antigen receptors expressed by pDCs that have been characterized to capture antibodies coupled to antigen complexes, process and present these antigens include human BDCA-2, DCIR, BST-2, FcRs and mouse SiglecH. However, the natural ligands of most of these receptors have not been identified and therefore evidence is lacking for their capacity to present natural ligands to T cells.

Plasmacytoid DCs and mDCs differ dramatically in MHC II-antigen presentation. Myeloid DCs constitutively produce and deliver new MHC II-antigen to the plasma membrane, while removing pre-existing ones through endocytosis and endosomal degradation (141). Upon activation, mDCs upregulate the synthesis of MHC II molecules and preferentially deliver them to foreign antigen-containing endosomes (84, 143, 144). Shortly thereafter, MHC II synthesis and the MHC II-antigen complex turnover rate is downregulated during mDC maturation. This allows the stabilization of MHC II expression and the maintenance of acquired antigen presentation. In contrast, pDCs do not downregulate MHC II synthesis and antigen loading. Therefore, pDCs lack the capacity to accumulate and present exogenous antigens generated after activation (141).

Activated pDCs can prime both TH1 and TH2 immune responses depending on the maturation status of the pDC and the nature of the antigen (145). In response to TLR-7/-9 ligands and viruses, pDCs produce large amounts of IFN-α and induce TH1 differentiation (109). Mouse pDCs produce IL-12, which also induces TH1 differentiation. In tolerogenic microenvironments (peyers patches),

virus stimulated pDCs can stimulate naïve T cells to produce IFN-γ and IL-10 (T regulatory cells), whereas human pDCs activated in non-IFN-inducing conditions of IL-3 and CD40L stimulation were found to skew the T cells towards TH2 responses (11). In addition to their ability to induce TH1 and TH2 responses, a tolerogenic function of pDCs has been suggested. Freshly isolated pDCs can anergize T cells and induce regulatory and suppressive activity in T cells (39, 139). Furthermore, thymus or fetal lymph node pDCs can induce tolerance or T regulatory cells (146). Plasmacytoid DCs have been recently shown to produce indoleamine 2, 3-dioxygenase (IDO), which may be a contributing factor to their ability to induce tolerance (147).

Activated pDCs can also prime $CD4^+$ and $CD8^+$ antigen specific responses *in vivo* and *in vitro* (136, 145, 148, 149). Virus stimulated pDCs have been reported to up-regulate maturation markers and prime $CD8^+$ T cells *in vivo* (149), while CpG activated pDCs were reported to activate $CD8^+$ memory T cells (150). In mDC depleted mice, ovalbumin (OVA) -peptide loaded pDCs have the ability to prime $CD4^+$ OVA-specific TCR transgenic T cells in lymph nodes (151). Finally, there is also evidence for pDC influence on humoral immunity. The influence of pDC secreted IFN-α on mDCs was found to strongly enhance the antibody response to antigen, resulting in class-switching and development of immunological memory (152). Furthermore, secretion of IFN-α and IL-6 by virus-stimulated pDCs induces differentiation of human B cells into Ig-secreting plasma cells (116). Thus, the interaction between pDCs and adaptive immune cells depends on pDC activation status and the nature of the antigen stimulus.

1.10. Role of pDCs in diseases

The ability of pDCs to produce unparalleled levels of IFN-α, amongst other cytokines and its ability to directly interact with other immune cells has prompted many investigators to dissect the role of pDCs in disease mechanisms. The discovery of mouse pDCs and the development of antibodies to pDC-specific markers has greatly aided in this process. There are a number of reports implicating the role of pDCs in the etiology of human viral and autoimmune diseases. The role of pDCs in anti-tumor responses remains elusive. However, there have been recent reports attempting to address this issue.

1.10.1 Viral diseases

Many studies have concentrated on the role of pDCs and their production of IFN-α in resistance to viral infections. Different viral infection models in mice are currently used to study pDC functions and their implications for immune responses. Plasmacytoid DC studies in the mouse model have typically involved either the use of knockout mice with disruptions of genes specific to particular components of the pDC signaling pathways or by depletions of pDCs *in vivo*. Since IFN-α is a crucial factor in host antiviral defense, many mouse studies have discovered the functional redundancy of mechanisms leading to IFN-α production. IRF-7 is comman to many downstream viral receptor pathways and would therefore likely play a bigger role in type I IFN production. Deletion of TLR-9 and MyD88 has fewer effects on resistance to HSV infection than deletion of IRF-7 (83). In a study by Ciavarra et al. (153), *in vivo* depletion of pDCs by mAb lead to decreased priming and clonal expansion of naive, virus-specific

$CD8^+$ T cells in mice infected with Vesicular stomatitis virus (VSV) even though the majority of the pDC-depleted mice were able to clear the virus infection. Lund et al. have recently demonstrated that pDCs are recruited to the vaginal mucosa in HSV-2 -infected mice and function there as innate antiviral effector cells, effectively reducing the viral load (154). In a murine Respiratory syncytial virus (RSV) model, pDCs were recruited to the lung and were able to inhibit RSV replication and reduced pulmonary inflammation and airway hyper responsiveness, thus emphasizing the role of pDCs in this compartment (155). TLR-3 and TLR-9-deficient mice have decreased resistance to infection with MCMV associated with decreased type 1 IFN and IL-12 production. In agreement with this finding, MyD88-deficient mice are more susceptible to MCMV then TLR-3 or TLR-9-deficient mice, suggesting either redundant or complementary defence (123).

Several lines of evidence have suggested that like $CD4^+$ T cells, decreased IFN-α producing capacity by PBMCs from HIV^+ patients correlates with disease progression and susceptibility to opportunistic infection. This is a direct result of decreased circulating pDC numbers and decreased IFN-α production by HIV^+ patients (156). Similarly, decreased pDC numbers and IFN-α secretion have been reported for acute Dengue virus and acute and chronic Hepatitis B and Hepatitis C (157, 158). However, in all these cases, the underlying factors contributing to reduced pDC numbers and function have not been dissected and are equally likely to be a cause or consequence of the disease.

1.10.2. Autoimmune diseases

A number of groups have reported on the immunopathology inflicted by pDCs. Systemic lupus erythematosus (SLE) is by far the best studied autoimmune disorder associated with pDCs. Many patients with SLE have decreased levels of circulating pDCs, but increased levels of redistributed skin pDCs that secrete abundant IFN-α into the skin and serum (159). IFN-α production in SLE is initiated by the impaired clearance of apoptotic cells, generating serum antibody-DNA or RNA complexes which are subsequently endocytosed via FcγRIIa on pDCs and translocated to intracellular TLR-9-containing endosomal compartments, resulting in the production of IFN-α (160, 161). The pDC-derived IFN-α then acts on mDCs to further trigger T cell-mediated autoimmunity and the promotion of B cell differentiation into plasma cells that produce auto-reactive antibodies. In addition to FcR mediated uptake of self nuclei acids in autoimmune diseases, Tian et al. implicate the pDC receptor RAGE in complexing with HMGB1, a nuclear DNA-binding protein released from necrotic cells complexed with CpG-ODN and their subsequent interaction with TLR-9/MyD88 to stimulate type I IFN production (162). Moreover, they demonstrate that aberrant IFN-a production by PBMC in serum from SLE patients can be inhibited upon addition of blocking mAb to HMGB1 and RAGE (162).

Similar to SLE, a variety of other autoimmune and inflammatory diseases are associated with dysregulation of pDC IFN-α production. It has been suggested that Sjogren's syndrome, a disease characterized by dysfunctional secretory glands and mucosal membranes is due to antibody-RNA complexes that

activate pDC IFN-α production through TLR-7 recognition (163). Psoriasis is another autoimmune disorder characterized by IFN-α production by skin-infiltrating pDC (164, 165). The natural ligand stimulating pDCs in psoriasis has yet to be identified. However, it has been demonstrated that topical treatment with imiquimod (TLR-7 synthetic agonist) further exacerbates psoriatic lesions (164-167). Recent work conducted by Lande et al. provides evidence for the role of LL37, an endogenous antimicrobial peptide in inducing psoriatic skin lesions. They demonstrate that LL37 binds to self-DNA and forms aggregated structures via ionic interactions that are delivered to TLR-9 containing endosomes and trigger massive IFN-α production (168). Increased expression of LL37 has also been reported in the mucosa of inflammatory bowel disease (169) and in the synovial of rheumatoid arthritis (170).

In addition to SLE and psoriasis, a variety of other autoimmune diseases are associated with dysregulation of pDC/IFN-α including type I diabetes, Hashimoto's disease, dermatomyositis and rheumatoid arthritis (171). Therefore, therapeutic approaches to modulate the pDC IFN-α response may be beneficial in the treatment of these diseases.

1.10.3. Cancer

Although most studies have focused on the role of type I IFN in viral and autoimmune disease, recent reports suggest that pDCs are also involved in tumour immunity, as evidenced by identification of pDCs in several experimentally induced mouse cancers and human cancers including melanoma and head and neck cancer. These studies hypothesize that pDCs may play a crucial role in

provoking cancer immunity via secretion of IFN-α, maturation of mDCs and enhancement of T cell priming. While cancer has been associated with dysfunctional myeloid differentiation resulting in decreased mDC maturation and accumulation of heterogeneous myeloid precursors (myeloid-derived suppressor cells) that suppress the immune system (172), no such inhibition of pDC differentiation by cancer has thus far been revealed.

Whereas many studies have focused on mDC induction of antitumor immune responses, the role of pDCs and its interaction with other cells in antitumor immunity has not been addressed. Lou et al. immunized mice with a mixture of activated pDCs and mDCs followed with subsequent tumor challenge. Mice immunized with the DC mixture demonstrated an enhanced antitumor response compared to mice immunized with either DC subset alone. T cell activation against tumor challenge was found to be dependent on MHC class I expression on mDCs, but not pDCs. This suggests that pDCs enhanced the ability of mDCs to present Ag to T cells (173).

A study by Hartmann et al., demonstrated the infiltration of pDCs into tumor tissues of patients with Head and neck squamous cell carcinoma (HNSCC). They found that pDCs isolated from single cell suspensions of primary HNSCC tissue were functionally unable to secrete IFN-α in response to TLR-9 stimulation by CpG. Moreover, they encountered down-regulation of TLR-9 expression. However, in the tumor-draining lymph nodes, there was less suppression of IFN-α production and this was associated with increased activation of $CD4^+$ and $CD8^+$ T cells (174).

In a study conducted by Palamara et al., imiquimod treatment lead to either a complete or significant reduction of experimentally implanted tumors and this correlated well with the number of recruited pDCs, suggesting that imiquimod uptake through TLR-7 induces a strong inflammatory response that mediates antitumor effects through stimulation of the host's immune system and/or by direct effect on tumor cells (175).

Recent reports model pDC antitumor immunity on SLE pathogenesis. In SLE, the impaired clearance of dead apoptotic cells generates DNA-immune complexes containing CpG, which activates pDCs and B cells through TLR-9. The activated pDCs produce IFN-α, which in turn activates mDC-induced T cell priming. Activated B cells differentiate into plasma cells, producing polyclonal antibodies. In cancer immunity, the induction of massive cell death by breaking up solid tumors through primary system chemotherapy results in release of tumor associated antigens and cell fragments containing hypomethylated CpG, which may be endocytosed by pDCs, channeled to TLR-9, resulting in the production of pro-inflammatory cytokines and type I IFN. The production of type I IFN can subsequently activate mDCs and prime T cells (176).

2. Natural killer cells

The immune system uses both innate and adaptive responses to recognize and clear pathogens from the host. The innate response provides initial control over invading pathogens using antigen non-specific mechanisms and primes the adaptive immune response (63, 177). Natural killer (NK) cells are large, granular, bone marrow-derived lymphocytes that discriminate between normal healthy cells

and abnormal cells by using a sophisticated repertoire of cell surface receptors that control their activation, proliferation and effector functions (94, 178, 179). These receptors are encoded by germline genes that do not require somatic recombination and thus categorize NK cells as specialized effector cells of the innate immune system. As integral members of the innate immune system, NK cells are involved in direct killing of cells displaying abnormalities linked to infection, malignancy or transplantation (94, 179, 180). Although NK cells lack the ability to generate antigen-specific receptors, they share many functional aspects with T cells. Like $CD8^+$ cytotoxic T lymphocytes (CTLs), NK cells employ a killing mechanism involving perforin and granzymes. The cytokines secreted by NK cells are also crucial to the activation of a wide variety of immune cell types and in controlling certain viral, bacterial and parasitic infections. Similar to CTLs and $CD4^+$ Th1 cells, NK cells share a similar pattern of cytokine secretion with IFN-γ being the predominant cytokine (94). Individuals with NK cell defects best illustrate the crucial role played by these important cells in the immune system (181-183). Viruses of the herpes family including varicella-zoster, CMV, herpes simplex, Epstein-Barr as well as the papilloma family of viruses chronically and recurrently infect these patients. These uncontrolled infections persist despite the presence of a normal adaptive immune system. These normally benign infections are fatal in NK cell-deficient individuals. Defective NK cell functions have also been linked with HIV disease progression (184). In other conditions, exaggerated NK cell responses have been implicated in the etiology of graft rejection, graft-versus-host disease and autoimmune

disorders. Thus, is it both essential and necessary to tightly control NK cell function (94).

2.1. Major histocompatibility complex molecules

The MHC multigene family is involved in antigen presentation and includes genes encoding all MHC class I and class II molecules (185). The majority of NK cell receptors families have been identified as recognizing both classical MHC class I molecules (MHC-Ia) and non-classical MHC class I molecules (MHC-Ib) (186). The MHC-Ia complex consists of covalently linked 45-kDA heavy chain, 12-kDA light chain, β_2-m (β_2-microglobulin) and a short peptide antigen. In humans, there are 3 MHC-Ia genes called HLA-A, -B and -C; and in mice – H-2K, H-2D and H-2L (187). MHC-Ia molecules are extremely polymorphic and are widely expressed. Most MHC-Ia peptides are derived from self or intracellular microbial proteins that are digested by the proteosomes, pumped into the endoplasmic reticulum, loaded into the peptide-binding groove and presented on the cell surface membrane for T cell receptor (TCR) and NK cell receptor recognition (187). There are also many MHC-Ib molecules in both humans and mice (188). In contrast to MHC-Ia, MHC-Ib molecules have few alleles in the population and have diverged from MHC-Ia molecules to perform diverse functions (188). The human and mouse CD1 MHC-Ib molecules, for example, bind glycolipids and lipopeptides (189, 190). Unlike MHC class Ia and Ib, no clear evidence exists for NK receptor recognition of MHC class II molecules (94).

2.2. NK cell recognition

NK cell recognition involves the initial binding to potential target cells, creation of an immune synapse whereby interactions between activating and inhibitory receptors with ligands available on the target take place, and the integration of signals transmitted by these receptors, which determines whether the NK cell detaches and moves on or stays and responds. NK cells respond by reorganizing and releasing the cytotoxic granules perforin and granzyme; and by transcribing and secreting cytokines such as IFN-γ (94, 178, 179, 191). NK cell recognition was initially described to be non-MHC-restricted because of their ability to lyse target cells that either lacked MHC or expressed various allogeneic MHC molecules (192). However, it was subsequently found that NK cells were actively inhibited from responding to certain tumor cells that express specific MHC class I (193). The ability of NK cells to recognize and kill normal host hematopoietic cells that lack MHC class I was subsequently supported by demonstrating NK cell-dependent rejection of bone marrow cells from syngeneic mice deficient in β2-microglobulin, a requisite subunit of class Ia MHC molecules (194, 195). The revised missing-self hypothesis explains the actions following NK receptor-ligand interaction. "NK cells patrol for abnormal cells that lack MHC class I or overexpress ligands for activating NK cell receptors" (94). Inhibitory receptors serve to regulate and dampen signals transmitted through activating receptors and it is the balance between activating and inhibitory signal that dictates target cell fate (94).

2.3. The leukocyte receptor complex and natural killer complex

To date, two NK receptor clusters have been characterized in all mammals. The leukocyte receptor complex (LRC) and the natural killer complex (NKC) contain two distinct structural families of NK cell receptors responsible for all NK activity: the Immunoglobulin (Ig) –like superfamily and the C-type lectin superfamily, respectively (196).

The human LRC is located on chromosome 19 and contains 15 killer cell Ig-like receptor (KIR), 1 leukocyte Ig-like receptor (LIR) and 2 leukocyte-associated Ig-like receptor (LAIR) genes (196). Different orders of species preferentially use one gene superfamily over the other to regulate NK activity. Humans preferentially use KIR receptors that are specific for classical MHC class I molecules (HLA-A, -B, -C) (197, 198). The mouse LRC (chromosome 7) contains 10 paired Ig-like receptors (PIRs) and 1 LAIR. They do not contain any KIRs or LIRs in their LRC, although 2 KIRs are located on chromosome X (196).

The human NKC is located on chromosome 12 and contains 15 C-type lectin receptor genes (196). In rodents, the NKC is located on mouse chromosome 6 and on rat chromosome 4 (199). The expanded mouse NKC contains 3 major C-type lectin superfamily receptors (Nkrp1 and NKG2/CD94, Ly49) (94, 178). The NKR-P1 family of type II transmembrane glycoproteins contain 6 known members and shows remarkable conservation in gene number and order across different mouse strains (200). Notably, NKR-P1 recognizes Clr ligands that are themselves type II transmembrane glycoproteins of the C-type lectin superfamily encoded by genes within the mouse NK complex. The human NKC contains a single NKR-P1 gene (200, 201). The mouse CD94/NKG2

receptors are specific for non classical MHC class I molecules (94). A single CD94 gene is closely linked to 3 NKG2 genes in mice. Similar to the NKR-P1 receptors, the NKG2 genes exhibit limited polymorphism and allelic variation. Humans contain 1 CD94 gene and 4 NKG2 genes (196). There is only one member of the Ly49 multigene family – Ly49L (a non functional molecule caused by a point mutation) found in humans (202), as opposed to multiple homologous Ly49 receptors in rodents. The Ly49 family of NK receptors is mapped on mouse chromosome 6 and rat chromosome 4 (199).

KIR and Ly49 genes represent a classic example of convergent evolution. The two gene families have evolved separately to achieve similar functions. Both the Ly49s and KIRs are highly polygenic and polymorphic. In addition, they share similar gene regulation, ligand binding specificity and intracellular signaling cascades (203).

2.4. Ly49 receptor family

Structurally, the Ly49 genes are members of the C-type lectin super family and encode type II transmembrane glycoproteins that are expressed as disulfide-linked homodimers (94, 178). Each homodimer chain is composed of a C-type lectin-like domain (CTLD) connected to the cell membrane by an α-helix stalk region of approximately 70 amino acids (204) (Figure 2). Despite the evolution of Ly49 receptors from C-type lectins, carbohydrates do not appear to be recognized by Ly49s (94).

To date, 23 Ly49 transcripts have been determined within the NKC, Ly49A through Ly49W. Of the 23 existing Ly49 NK cell receptors, 13 are

inhibitory (Ly49A, B, C, E, F, G, I, J, O, Q, S, T and V), based on the presence of intracytoplasmic ITIM or functional data. Ly49D, H, L, M, P, R, U and W are presumed to be activating. Ly49 receptors can be divided into both activating and inhibitory types (Figure 2). Activating Ly49s contain a positively charged arginine residue in its transmembrane domain that interacts with a negatively charged aspartic acid residue in the DAP-12 adaptor molecule (191). In the absence of DAP-12, the activating Ly49 receptors are not stably expressed on the cell surface (205), although recent evidence suggests that Ly49H and Ly49D can also associate with the related DAP-10 molecule (206, 207). However, no consensus has been reached on the functional consequences of this activating Ly49-DAP-10 association. DAP-12 contains a single ITAM motif (208). The ITAM is defined by the consensus sequence Asp/Glu-x-x-Tyr-x-x-Leu/Ile x6-8 Tyr x x Leu/Ile, where x is any amino acid with 6-8 amino acids between the two Tyr xx Leu/Ile elements (209). The ITAM in DAP-12 has been shown to interact with Syk/Zap 70 kinases and mediates downstream phosphorylation and signaling cascades (205). The only definitively known activating Ly49 ligand is the interaction between Ly49H and the MCMV encoded MHC-like m157 molecule (210). Other groups have shown some level of interaction between activating Ly49 and H-2 molecules. Ly49D has been shown to recognize H-2D^d in *in vitro* cytotoxicity assays (211-213). Likewise the activating Ly49P and Ly49W receptors in NOD mice also have been shown to recognize H-2D^d ligands (214, 215), but the biological significance of this interaction is unclear at present. The enigmatic interactions between activating Ly49s and inhibitory Ly49 ligands are thought to represent weak remnants of biological cross reactivity. Insight into this

mystery may come from other NK receptors outside of the Ly49 family. The NKG2D receptor binds to a MHC related stress molecules expressed on infected or abnormal cells (216, 217). This may also hold true for yet unidentified activating Ly49 receptors and their ligands. Presumably, binding of ligand to activating receptor leads to activation of cytotoxic mechanisms of the NK cell and secretion of cytokines.

Inhibitory Ly49s, on the other hand, contain an ITIM in their transmembrane domain. The ITIM consensus sequence consists of Ile/Val/Leu/Ser-x-tyr-x-x-Leu/Val, where x denotes any amino acid. Upon ligand binding, the two ITIMs of the inhibitory receptor (lectin dimer) become phosphorylated by a membrane proximal kinase. This in turn recruits a phosphatase, src homology-2 phosphatase (SHP)-1 or SHP-2 through the SH2 domain (94). Src homology 2 domain-containing inositol-5-phosphatase (SHIP) recruitment has also been reported (218). This results in general dephosphorylation of the cellular signaling components and inhibition of cell activity. A recent finding by Yu et al. identified the signaling molecule β-arrestin 2 to associate with the inhibitory human KIR2DL1 NK receptor and facilitate the recruitment of SHP-1/-2 and downstream inhibitory signaling (219). The exact targets of SHP-1/-2 are unknown and are presumed to be membrane proximal kinases. The end result is general dephosphorylation of the cellular signaling components and inhibition of cell activity (191). The ligand binding affinity of many inhibitory NK cell receptors has been defined. They are MHC class Ia molecules, non classical (class Ib) MHC molecules and viral MHC mimics (94).

Clues into the structural binding between Ly49 and H-2 molecules have been obtained from mutational analyses and crystal structure studies. The crystal structure of Ly49A receptor bound to the H-2D^d molecule has been described (220). The homodimeric Ly49A receptor binds asymmetrically to H-2D^d molecule at two distinct sites. Steric hindrance prevents the dimeric Ly49A from binding bind two MHC-Ia molecules. Site 1 involves contact with the α1 and α2 domains of H-2D^d and site 2 spans the α1, α2, α3 domains and β_2-m (221). In contrast, the binding complex of Ly49C-H-2K^b is very distinct from that of Ly49A-H-2D^d. The Ly49C homodimer interacts symmetrically and bivalently with two H-2K^b molecules, both on site 2 (222). The interface between Ly49-H-2 molecules offers an explanation for MHC specificity (204, 222). Ly49C mainly binds to H-2K^b along non-polymorphic residues of the MHC-Ia heavy chain and at only one contact point with β_2m, whereas β_2m contacts Ly49A at two distinct amino acid residues (222). In addition, there are more electrostatic interactions between Ly49A-H-2D^d compared with Ly49C-H-2K^b (186, 204). These factors explain MHC cross-reactivity differences between Ly49C and Ly49A (186, 204, 222). Often considered as a pan-MHC receptor, Ly49C recognizes H-2D^d, H-2D^k, H-2K^d and H-2D^b in addition to H-2K^b (222). In contrast, the specificity of Ly49A is restricted to H-2D^d and H-2D^k (220).

The traditional model of cell-to-cell communication is understood to involve *trans* interaction, which describes the interaction between cell surface receptors and ligands expressed on other cells. However, certain receptors can also engage ligands in *cis* or expressed in the plane of the same cell membrane

(223, 224). The *cis* and *trans* model could explain the observation that the level of surface expression of Ly49 receptors is lower in mice expressing self MHC class I molecules specific for that given Ly49 receptor (225). The molecular basis of *cis* vs. *trans* interactions between Ly49 receptors and H-2 molecules is currently unknown (223). Site-directed mutagenesis studies have described site 2 to be predominantly responsible for trans interaction between Ly49A and H-2D^{d} on opposing cells. Site 1 has been described to modulate lower affinity *cis* interactions between Ly49A and H-2D^{d} on the NK cell itself (220). Similarly, Doucey et al. suggested that the interaction at site 1 could account for *cis* association while *trans* interaction could take place at site 2 (226). Most recently, Held's lab provided structural and functional evidence revealing that *cis-trans* interactions of Ly49-MHC are mediated by two distinct receptor conformations (223). The back-folded conformation allows the receptor to engage MHC-Ia in *trans*, while the extended conformation allows for *cis* interaction. These receptor conformations are in turn mediated by the flexible Ly49 stalk region (223). The functional consequences of cis vs. trans interactions have been addressed by several current studies (223, 227). In back-to-back investigations by Held's group, various inhibitory Ly49 molecules has been shown to interact with their MHCIa ligands *in cis* because soluble MHC I tetramers bind inefficiently to inhibitory Ly49s unless the NK cells are rendered deficient for MHC I complexes (224). Further, *cis* interactions continuously sequester a considerable portion of the inhibitory Ly49A receptor, preventing efficient recruitment of Ly49A to the immunological synapse for H-2D^{d} interaction *in trans* (223, 227). This reduced number of Ly49A receptors that can functionally interact with H-2D^{d} *in trans* on

target cells explains the modest inhibitory capacity of Ly49A in H-2D^d expressing NK cells and renders Ly49A NK cells more sensitive to react to diseased host cells (227).

In addition to binding with the H-2 molecule, Ly49 binding requires the presence of a bound peptide with the H-2 groove (220, 221). However, no consensus has been reached over the biological significance of the peptide requirement (94). Peptide promiscuity has been shown for the Ly49A receptor, while peptide selectivity has been demonstrated for Ly49C (228, 229). However, both Ly49A and Ly49C have not been demonstrated to make any direct contact with bound peptide (220, 222). This is in stark contrast to T-cell receptor (TCR)-peptide-MHC I recognition. The TCR has been shown to bind the peptide:MHC I complex with direct interaction between various complementary-determining regions on the TCR with peptide in the antigen-binding site (230). This difference in binding between TCR and NK receptor recognition of MHC highlights the functional consequences of adaptive immune receptors and invariant, genome encoded receptors.

Four mouse Ly49 haplotypes have been completely sequenced to date, confirming both remarkably variable gene content and limited conservation (231) (Figure 3). In mice, there are three pairs of framework genes (*Ly49c-a*, *Ly49i-g* and *Ly49q-e*) that provide a scaffold within which regions of variable numbers of unique strain-specific genes reside (232). The human KIR haplotypes are similar to the Ly49s in complexity and in conservation (197, 198, 233). The smallest known Ly49 cluster exists in Balb/c mice, containing only eight genes (232). C57BL/6 mice possess 15 Ly49 genes with *Ly49h* being the most notable. This

stimulatory receptor directly recognizes the MCMV-encoded MHC-like *m157* gene product on virally infected cells (234, 235). This interaction provides MCMV resistance to C57BL/6 mice, while strains that lack *Ly49h* are susceptible (210, 234). The 129S6 strain contains 19 Ly49 genes (where *Ly49i2-o* are allelic variants of *Ly49c-a*) with two duplications of the *Ly49q-e* region, which leads to Ly49q2-ec2 and Ly49q3-ec1 gene pairs (236). However, *Ly49q2* and *Ly49q3* are considered pseudogenes because they do not contain exons 6 and 7 which encode the ligand binding domain. Lastly, the NOD strain contains the largest Ly49 gene set containing 21 Ly49 genes (231). This newly sequenced Ly49 cluster encodes the largest number of stimulatory Ly49 receptors, but this increased activation potential does not correlate with an expected elevation in NK function (200, 231, 232, 236, 237). However, the relatively large number of stimulatory Ly49 in NOD mice parallels the finding in human type I diabetics of increased number of activating KIRs compared to non-diabetic individuals (197, 231).

Of all the Ly49s, *Ly49q*$_1$ and *Ly49b* have the most distant homology to the other Ly49s. These two genes border each extremity of the cluster and are also the only two members that have no reported expression on NK cells but on pDCs (35, 238) and macrophages (239), respectively. The gene encoding Ly49B is separated by >0.8 Mega bases from the Ly49 gene cluster (200, 239). Therefore, the evolutionary pressure on these two Ly49s and likely their functions are different from the others due to this unique expression pattern. The phylogenic tree (Figure 4) of all the different Ly49s known from B6, 129S6 and Balb/c mouse strains shows the relatedness of the genes and allows grouping into nine subfamilies: D, L, A, G, C, H, E, Q, and B-like (240).

2.5. The mouse pDC receptor: Ly49Q

Located at the centromeric extremity of the *Ly49* cluster, *Ly49q*$_1$ is one of the most divergent members of the *Ly49* family. Despite its divergence, *Ly49q*$_1$ is highly conserved in the four different mouse haplotypes sequenced to date (231, 237) and the protein product has been detected in all mouse strains tested (238). This suggests an important and conserved function of the Ly49Q receptor. Similar to other Ly49s, Ly49Q is a type II C-type lectin transmembrane protein. Ly49Q contains an ITIM motif in the cytoplasmic tail and no positively charged arginine residue in its transmembrane domain, which are characteristics of inhibitory Ly49s expressed on NK cells. It has also been reported to associate with the phosphatase SHP-1 and SHP-2 after cross-linking of the receptor on Ly49Q-transfected macrophages (35). *Ly49q* is composed of 7 exons, which code for a 273 amino acid long protein. Exon 1 and a small part of exon 2 form the 5' untranslated region and comprise regulatory elements including promoter regions. Most of exon 2 codes for the intracellular domain containing the ITIM signaling motif, exon 3 codes for the transmembrane domain, exon 4 codes for the stalk domain and exons 5, 6 and 7 codes for the ligand-binding extracellular domain (35, 238).

In contrast to other members of the Ly49 family, Ly49Q cell surface expression is not found on NK, NKT and T cell subsets. Ly49Q was first reported to be expressed at low levels on a proportion of $Gr1^+$ bone marrow myeloid precursor cells, peripheral blood neutrophils ($Gr1^+$ $CD11b^+$) and IFN-γ producing macrophages (35, 238) Further investigation of Ly49Q expression identified a

cell population expressing significantly higher levels of Ly49Q than neutrophils or macrophages (35, 238). This cell population was originally defined as $CD11c^{+}B220^{+}Gr1^{low}$ and since then has been confirmed to represent pDCs (14). Ly49Q is expressed on all peripheral pDCs and almost all bone marrow pDCs. Ly49Q levels correlate well with pDC developmental maturation and receptor acquisition is further up-regulated by various stimuli, including IFN-α (35, 238). In some but not all inbred mouse strains, a subset of mDCs also expresses low levels of Ly49Q (35).

A role for Ly49Q in macrophage migration and phagocytosis has been suggested after Ly49Q cross-linking on activated macrophages and subsequent cytoskeletal rearrangement lead to formation of polarized filopodia and lamellopodia (238). On the subset of bone marrow pDCs that express Ly49Q, a developmental role for Ly49Q has been suggested. The acquisition of expression of Ly49Q on bone marrow cells is thought to be linked to the developmental maturation of pDCs as $Ly49Q^{-}$ precursor cells are unresponsive and defective in cytokine secretion (111).

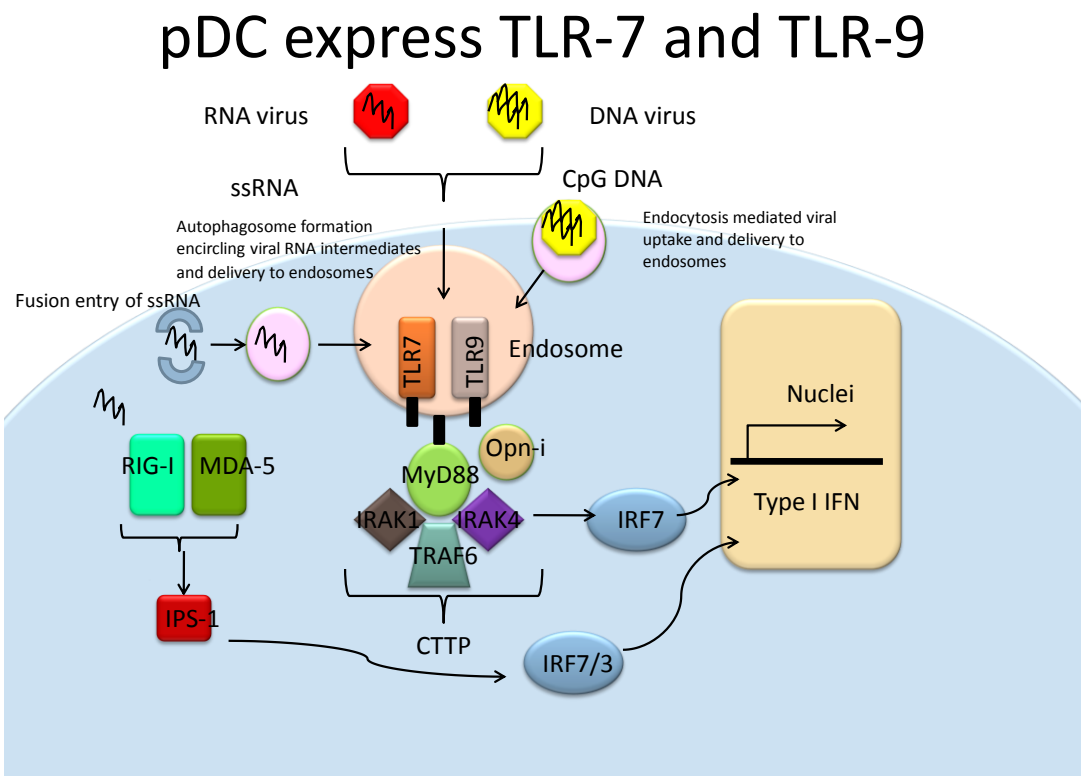


Figure 1. Signaling pathways in pDCs leading to production of type I IFN. Microbe enters the cell either through endocytosis or direct fusion with the cell membrane. Fusion of virus generates ssRNA intermediates that are delivered to TLR containing endosomes through the process of autophagy. Endocytosed viral particles are uncoated in the acidic endosome and the microbial DNA or RNA interacts with TLR-9 or TLR-7, respectively. MyD88 complexes with IRAK1, IRAK-4 and TRAF-6, which in turn is responsible for IRF-7 phosphorylation. IRF-7 is required for the transcription of type I IFN genes. Opn-i complexes with MyD88 and is crucial for type I IFN production. Viruses that enter the cytoplasm and replicate may activate the RIG-I/MDA-5/IPS-1 dependent pathways, resulting in IRF-3/-7 activation and type I IFN production.

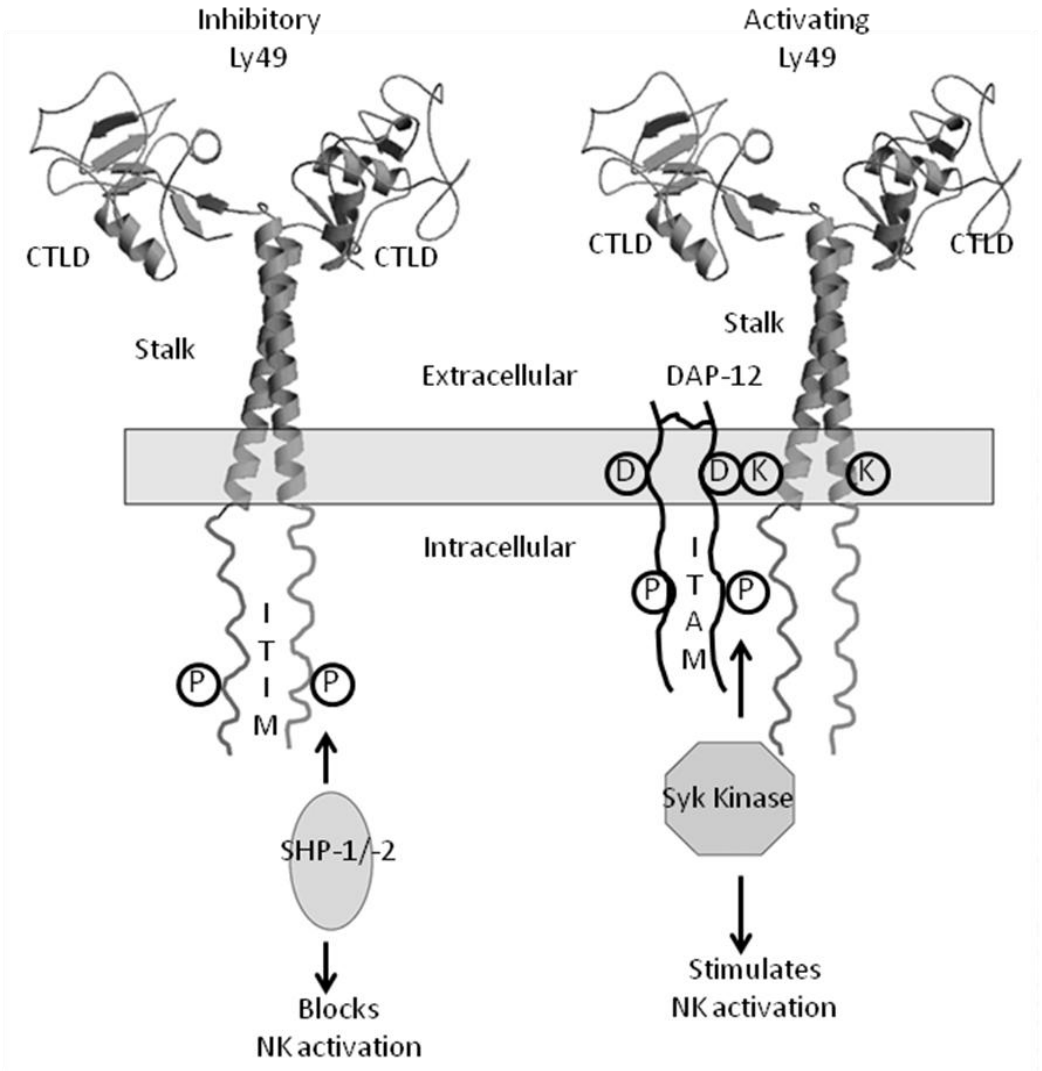
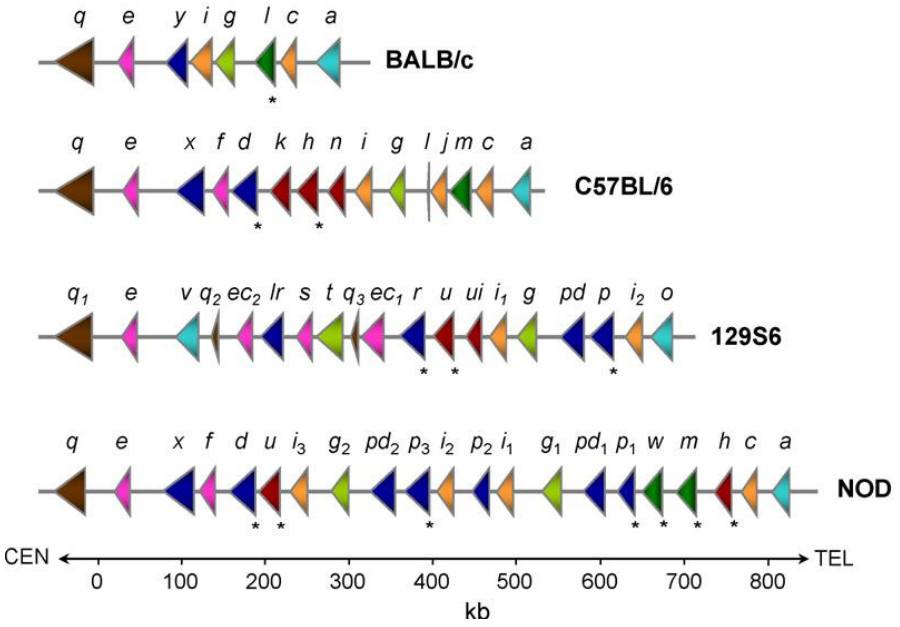


Figure 2: Inhibitory and Activating mouse Ly49 NK cell receptors. Ly49 NK cell receptors are 44 kDa type II homodimeric disulphide-linked transmembrane proteins consisting of a COOH terminal extracellular C-type lectin-like domain (CTLD), connected to the cell membrane by a stalk region of approximately 70 amino acids. Upon engagement, Ly49 inhibitory receptors recruit the tyrosine phosphatase SHP-1, which in turn inhibits NK cell activation. In contrast, engagement of Ly49 activating receptors results in recruitment of DAP-12, DAP-12 immunoreceptor tyrosine-based activating motifs (ITAM) phosphorylation, subsequent recruitment and activation of Syk kinase. This event initiates NK cell activation.



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

Figure 3: Comparison of gene content between four divergent mouse *Ly49* haplotypes. The Ly49 gene cluster is depicted for the BALB/c, C57BL/6, 129S6 and NOD inbred mouse strains. Total gene numbers are indicated along with S:I:ψ ratios = number of activating receptors, number of inhibitory receptors and pseudogenes. The orientation and relative size of the Ly49 genes are indicated by the direction and size of the triangles (kilobases). Genes encoding stimulatory Ly49 receptors are indicated with an asterisk (*) (Carlyle *et al.* 2008).

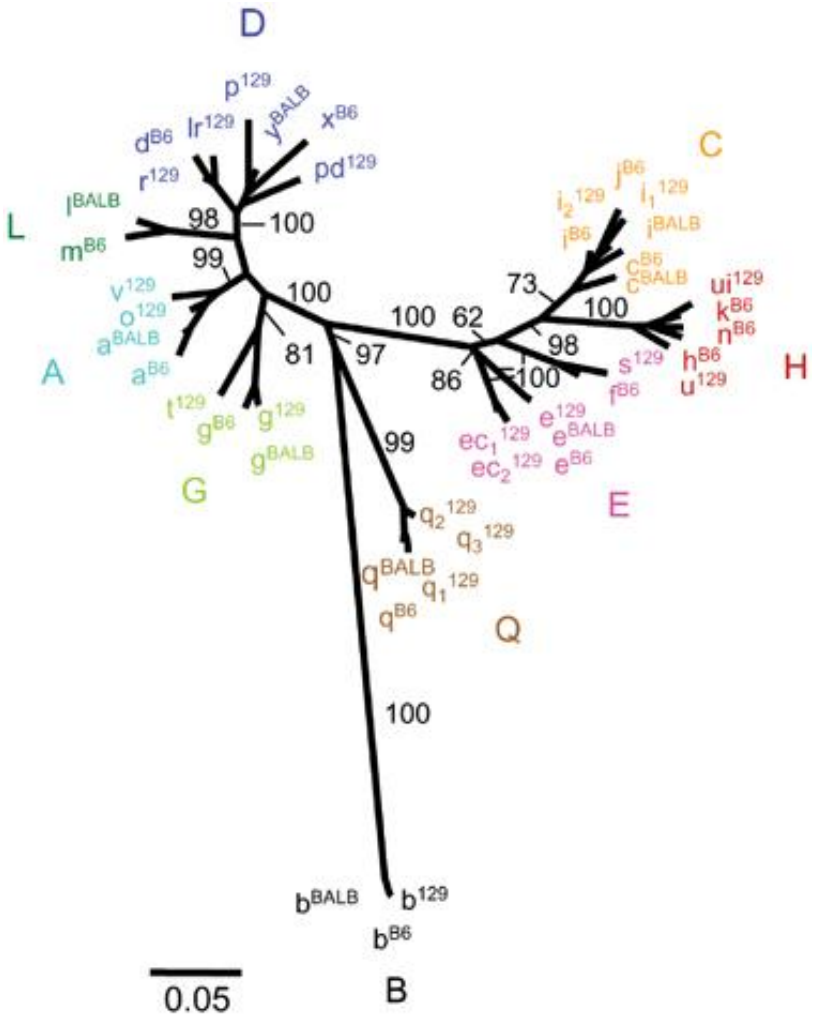


Figure 4: Phylogenic tree of Ly49 receptors. Nine different families of Ly49 can be distinguished by this analysis: D, L, A, G, C, H, E, Q, B. Each family is identified by a different color. A similar shade denotes a pair of activating and inhibitory receptors. (Makrigiannis *et al.* 2005).

RATIONALE AND OBJECTIVES OF THE RESEARCH

Prior to our investigation, both the specific ligand and function of Ly49Q on pDCs were unknown. All previous inhibitory Ly49 expressed on NK cells have been shown to recognize MHC class I molecules. Due to the homology of Ly49Q to NK Ly49 inhibitory receptors, the Ly49Q ligand is hypothesized to be a MHC class I or MHC class I-like molecule present on cells that interact with pDCs. A previous report has indicated that the ligand appears to be dependent on β_2-m for cell surface expression (238). Identifying the ligand for Ly49Q will allow for a more focussed approach to designing experiments for testing Ly49Q function in the Ly49Q-deficient mouse previously generated in our lab.

In terms of function, we hypothesize an inhibitory role for Ly49Q due to the presence of the ITIM-bearing cytoplasmic domain in the receptor and previous studies showing the recruitment of SHP-1/2 to the ITIM following receptor cross-linking (35). However, several lines of observation point to a potential stimulatory role for Ly49Q. As described by Toyama-Sorimachi and colleagues, macrophage migration and phagocytosis has been observed upon Ly49Q cross-linking on transfected macrophages, suggesting a stimulatory role for this receptor (35). Furthermore, recent studies of DAP-12 coupled pDC receptors using cross-linking antibodies have shown inverse phenotypes (Siglec-H, Nkp44) (33, 49) Thus, we cannot rule out the possibility of a stimulatory role for Ly49Q in pDC function.

The specific objectives of this research project are as follows:

1) To identify the natural ligand for Ly49Q.
2) To characterize the functional consequences of Ly49Q:H-2K^{b} interaction on pDCs.
3) To determine the functional consequences of receptor absence in the Ly49Q-deficent mouse.

PREFACE TO CHAPTER 2

The unique and conserved expression pattern of Ly49Q on pDCs suggests an important role of this receptor during the innate immune response (35, 240). Previous *in vitro* studies have shown a generalized increase in protein phosphorylation and macrophage cytoskeletal rearrangement after Ly49Q cross-linking (35). With the endogenous Ly49Q ligand unknown, these investigations were conducted by ligation of the receptor with antibody. Determining the precise ligand specificity of the Ly49Q receptor will greatly direct functional analyses into the role of Ly49Q in modulating pDC immune function.

Because the known ligands for ITIM-containing Ly49 receptors are MHC class I molecules, the Ly49Q ligand was speculated to be an MHC class I molecule, although MHC-related and MHC–non-related molecules of host or viral origin remained possible due to previous ligand identifications of activating Ly49 receptors and other NKC-encoded receptors.

Herein, we used receptor transfected reporter cells and *ex vivo* sorted pDCs to demonstrate that Ly49Q is triggered by an MHC class I molecule. We examined the basic parameters of the putative ligand, including haplotype specificity and by systematically excluding other candidates, reached the conclusion that the ligand is indeed the MHC class I molecule H-2K^{b}.

CHAPTER 2

Recognition of H-2K^b by Ly49Q suggests a role for class Ia MHC regulation of plasmacytoid dendritic cell function

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Running Title: H-2K^b is a ligand for Ly49Q on pDC.

1. ABSTRACT

Ly49Q is a member of the polymorphic Ly49 family of NK cell receptors that displays both a high degree of conservation and a unique expression pattern restricted to myeloid lineage cells, including plasmacytoid dendritic cells (pDCs). The function and ligand specificity of Ly49Q are unknown. Here, we use reporter cell analysis to demonstrate that a high-affinity ligand for Ly49Q is present on H-2^b, but not H-2^d, H-2^k, H-2^q, or H-2^a-derived tumor cells and normal cells *ex vivo*. The ligand is peptide-dependent and MHC Ia-like, as revealed by its functional absence on cells deficient in TAP-1, β_2m, or H-2K^{b}D^b expression. Furthermore, Ly49Q is specific for H-2K^b, as the receptor binds peptide-loaded H-2K^b but not H-2D^b complexes, and Ly49Q recognition can be blocked using anti-K^b but not anti-D^b mAb. Greater soluble H-2K^b binding to ligand-deficient pDCs also suggests *cis* interactions of Ly49Q and H-2K^b. These results demonstrate that Ly49Q efficiently binds H-2K^b ligand, and suggest that pDC function, like that of NK cells, is regulated by classical MHC-Ia molecules. MHC recognition capability by pDC has important implications for the role of this cell type during innate immune responses.

2. INTRODUCTION

Plasmacytoid dendritic cells (pDCs), also known as interferon-producing cells (IPCs), are a specialized subset of DC especially suited for initiating immune responses to viruses. Production of abundant IFN-α levels as well as IL-12 and other cytokines by pDC has direct anti-viral effects on infected cells, and further activates effector cells such as natural killer (NK) cells to destroy infected targets. Plasmacytoid DC express TLR7 and TLR9, allowing for recognition of viral single-stranded RNA and CpG-containing double-stranded DNA, respectively, in endosomal compartments (241). Likewise, expression of TLR9 by pDC and myeloid dendritic cells (mDC) is necessary for protection against mouse cytomegalovirus (MCMV) challenge (80, 242). In addition to activating NK cells via type I interferon, pDC also augment $CD8^+$ T cell cytotoxicity (148). Furthermore, activated pDC upregulate T cell costimulatory molecules such as CD86 and class II MHC molecules, allowing them to present antigenic peptides and stimulate $CD4^+$ T cell function (13). In turn, pDC have been implicated in promoting mDC maturation and B cell differentiation to antibody-producing plasma cells (241).

Plasmacytoid DC are identifiable by their plasma-cell-like morphology and expression of specific markers including Siglec-H (33), a DAP12-associating transmembrane receptor, and bone marrow stromal cell antigen 2 (BST2), recently shown to be the antigen recognized by the pDC-specific mAbs, 120G8 and mPDCA-1 (31). Interestingly, pDC also express Ly49Q, a member of the lectin-like Ly49 family of NK cell receptors specific for class I MHC molecules.

Ly49Q is not expressed on NK cells or T cells, but is present on adult Gr-1$^+$ splenocytes and bone-marrow cells, CD11b$^+$Gr1$^+$ peripheral blood cells, and most peripheral pDC, where it appears to be a differentiation marker (35, 243). In contrast to mature pDC in the spleen and lymph nodes, developing Ly49Q$^+$ and Ly49Q$^-$ pDC subsets can be identified in the bone marrow. Here, Ly49Q$^-$ pDC are thought to represent precursor cells and have a more limited ability to produce cytokines in response to TLR stimulation (111, 238).

Unlike Ly49Q, most other Ly49 proteins are expressed on NK, T, and NKT cells, except perhaps the divergent Ly49B, which is expressed on macrophages (239). All known ligands of the mouse inhibitory Ly49 receptors are class Ia MHC molecules (244), while certain rat Ly49 receptors have been shown to recognize class Ib MHC ligands (245). *Ly49* genes and alleles show great variation among different inbred mouse strains, unlike the more limited diversity observed for adjacent lectin-related multi-gene clusters, such as the *Nkrp1/Clr* loci (246). The three sequenced mouse *Ly49* haplotypes, 129S6, B6, and BALB/c, possess 19, 15, and 8 *Ly49* genes, respectively (232). However, despite such variation, *Ly49q* is present in all three haplotypes. *Ly49q* represents one part of three independent pairs of framework genes (*Ly49a* and *g*, *c* and *i*, and *e* and *q*), which are conserved among the known mouse *Ly49* haplotypes (237). The single characterized rat *Ly49* haplotype (BN/SsNHsd/MCW) contains a total of 36 *Ly49* genes, including a cluster of *Ly49q*-related genes (247-249). Whether rat *Ly49q*-related genes are also expressed in pDC is unknown. In the mouse, *Ly49q* is extremely well conserved in comparison to the other known *Ly49* genes, which are allelically diverse. In terms of relatedness to other *Ly49* genes, *Ly49q* and

Ly49b are the most divergent *Ly49* family members and are phylogenetically grouped in their own subfamilies by sequence alignment analysis (240).

Despite this divergence, Ly49Q contains an ITIM in its cytoplasmic domain, predicting it to be an inhibitory receptor by analogy to Ly49 expressed on NK cells. Consistent with this, co-immunoprecipitation experiments have shown that Ly49Q can associate with SHP-1 and SHP-2 (35). Interestingly, Ly49Q ligation of IFN-γ-treated RAW macrophage-like cells with plate-bound anti-Ly49Q mAb results in cell adherence and elongation, suggesting that Ly49Q may have some stimulatory function. As the identity of the cognate ligand recognized by Ly49Q is unknown, the role of Ly49Q in regulating pDC function has remained elusive to date.

In this study, we utilize a modification of the BWZ reporter cell assay (201, 246, 250) to investigate the expression of potential Ly49Q ligands. A series of experiments determined that various C57BL/6 (B6) and 129-derived tumor lines as well as *ex vivo* cell populations express a ligand for Ly49Q. Moreover, ligand expression was found to be peptide- and β_2m-dependent, and absent on cells derived from H-2K^{b}D^b double-deficient mice. Identification of H-2K^b as a ligand for the Ly49Q receptor suggests that classical MHC-Ia molecules regulate the function of pDC, in addition to NK and T cells.

3. MATERIALS AND METHODS

Mice

C57BL/6/J (B6), 129S1, 129X1, B6.129P2-*B2m*^{tm1Unc}/J (β_2m-KO), and B6.129S2-*Tap1*^{tm1Arp}/J (TAP-KO) were purchased from Jackson Laboratories (Bar Harbour, Maine). *H-2Kb*^{tm1}*H-2Db*^{tm1} (K^bD^b-KO) mice on a B6 background were purchased from Taconic (Hudson, NY). C3H (Dr. Q. Zhu), A/J (Dr. C. Deschepper), and FVB (Dr. A. Veillette) mice were all kind gifts of colleagues from the IRCM (Montréal, QC). Mice were maintained and bred in the IRCM specific-pathogen free animal facilities.

Cells

BWZ.36 cells were obtained from Dr. N. Shastri (University of California, Berkeley, CA). B16 (melanoma, H-2^b), RMA (thymoma, H-2^b), RMA-S (MHC-deficient variant of RMA), YAC-1 (lymphoblast, H-2^a), BW5147 (thymoma, H-2^k) and A20 (B lymphoma, H-2^d) were generously provided by Dr. A. Veillette (IRCM). P815 (mastocytoma, H-2^d), EL-4 (thymoma, H-2^b), L929 (fibroblast, H-2^k), RAW309 (macrophage, H-$2^{b/d}$), and NIH/3T3 (fibroblast, H-2^q) were obtained from Dr. Stephen Anderson (SAIC-Frederick, MD). RMA-S-CD1d1 was a kind gift of Dr. Kevin Kane (University of Alberta, Edmonton, AB). C1498 (acute myeloid leukemia, H-2^b) was obtained from Dr. James Ryan (University of California, CA) and MC57G (fibrosarcoma, H-2^b) was obtained from Dr. W-K. Suh (IRCM). DC2.4 (dendritic cell, H-2^b) was a kind gift of Dr. K. Rock (Dana-Farber Cancer Institute, Boston, MA). T2 (human T-B lymphoblast hybrid) cells and the Qa1^b-transfected T2g37 (T2-Qa1^b) subline were a kind gift of Dr. M.

Soloski (Johns Hopkins University School of Medicine, Baltimore, MD). The B78H1TAP (B16 melanoma-derived, H-2^b) parental (TAP) and Q9TAP.13 Q9-expressing subline (TAP-Q9) were generously provided by Dr. I. Stroynowski (University of Texas Southwestern Medical Center, Dallas, TX). DC were isolated from collagenase (Roche Diagnostics, Laval, QC) treated splenocytes using anti-CD11c-conjugated microbeads (Miltenyi Biotec, Auburn, CA). Peritoneal exudate cells were isolated by PBS lavage.

Antibodies, MHC-reagents, and flow cytometry

Anti-Ly49Q mAb 2E6 was purchased from MBL (Japan) and biotinylated using a kit (Roche). Anti-Ly49Q mAb NS-34 was previously described (Toyama-Sorimachi et al., 2004). The H-2K^b:Ig and H-2D^b:Ig fusion proteins were purchased from BD Biosciences (Toronto, ON). H-2K^b:Ig was incubated overnight in the presence of four molar excess of ovalbumin SIINFEKL peptide (a kind gift Dr. A. Veillette, IRCM) in PBS at 37^{o}C. H-2D^b:Ig was similarly prepared with LCMV gp33 peptide KAVYNFATM (Sigma, Oakville, ON). Antibody or fusion protein coating of flat-bottom 96-well plates was performed in PBS at the indicated concentrations for 90 minutes followed by several PBS washes. FITC-conjugated affinity purified donkey anti-human IgG (H+L) (Jackson Lab., West Grove, PA) was used as a secondary antibody to reveal Ly49Q-Ig. All staining reactions included 20% normal rat serum and 10 ug/ml of 2.4G2 (anti-CD16/32) mAb for blocking non-specific binding. PE-conjugated H-2K^b/OVA tetramer contains human β_2m and was purchased from Beckman

Coulter (Fullerton, CA). Flow cytometry and analysis was performed using a FACsCalibur (BD Biosciences, Mountain View, CA).

Construction of the CD3ζ/NKR-P1B/Ly49Q chimeric receptor and BWZ assay

A pMSCV2.2-CMV-IRES-GFP (pMCIG) retroviral vector was modified to include the intracellular region of CD3ζ and the membrane proximal and transmembrane regions of NKR-P1B, as previously described (201). The extracellular domains of $Ly49Q^{BALB}$ and $Ly49Q^{B6}$ were amplified from splenocyte cDNA using primers Q-EC-Xho-for, CTC GAG AAC ATT TTG CAG TAT AAG CAA G and Q-EC-Not-rev, GCG GCC GC TTA ACT GTT GTT GGG GAG CG (restriction sites are underlined). PCR products were TOPO TA-cloned into pCR2.1 (Invitrogen Life Technologies), cut with *Xho*I and *Not*I, and then ligated in-frame adjacent to the CD3ζ/NKRP1-B cassette. BWZ.36 cells were transduced with retroviral supernatants (24-48 h) from transient triple-transfected 293T cells, as described previously (201), then sorted at day 3-4 following transduction. Stable BWZ transductants were analyzed using plate-bound mAb or cell mixtures, as previously described (201).

Preparation of Ly49Q-human IgG_1 fusion protein

The CD5 leader-IgG_1 vector, kindly provided by Dr. B. Seed (Dept. of Molecular Biology, Massachusetts General Hospital, Boston, MA), was employed for the production of a $Ly49Q^{B6}$ protein fused to the human IgG_1 constant region. A stop codon at the 3' end of the CD5 leader-IgG1 cassette was replaced with an *Eco*RI-cloning site by PCR-based mutagenesis. The extracellular region of $Ly49Q^{B6}$ was amplified by PCR using *Eco*RI-containing primers, digested with *Eco*RI, and

ligated into the CD5 leader-IgG_1 vector at the 3' end of the IgG_1 sequence. Ly49Q-Ig chimeras were collected as serum-free supernatants of CHO transfectants, and used after concentrating using ultrafilter membranes.

4. RESULTS

Construction of a Ly49Q-ligand reporter cell assay

To elucidate the expression of a cognate ligand for Ly49Q, we employed a modification of the BWZ colorimetric reporter cell assay (201, 246, 250). BWZ.36 cells containing a *lacZ* transgene under the control of an NFAT-inducible promoter (250) were stably transduced to express a chimeric protein consisting of the CD3ζ cytoplasmic tail and NKR-P1B CxCP-motif/transmembrane region (201, 246), and the extracellular domain of $Ly49Q^{BALB}$ or $Ly49Q^{B6}$. $Ly49Q^{B6}$ and $Ly49Q^{BALB}$ are allelic variants that differ at four and two amino acid substitutions in the stalk and lectin-like domains, respectively (237). Expression of the CD3ζ/NKR-P1B/Ly49Q fusion receptor was monitored using GFP fluorescence (driven by an IRES-GFP element in the retroviral cassette) (201). To ensure that the fusion receptor was expressed on the surface of BWZ.36 transductants and that proper folding had taken place, CD3ζ/NKR-P1B/Ly49Q-transduced BWZ.36 cells (hereafter referred to as BWZ.Ly49Q) were stained with the anti-Ly49Q mAb, 2E6. Flow cytometric analysis of sorted $BWZ.Ly49Q^{BALB}$ and $BWZ.Ly49Q^{B6}$ transductants revealed that both cell lines expressed the fusion protein at the cell surface (Fig. 1*A*). While high surface levels of the fusion receptors were difficult to attain, both cell lines expressed similar but low levels of GFP (Fig. 1*A*). Notably, this low level of fusion-receptor and IRES-GFP expression has been observed before, and may be related to co-expression of a low-level or weak Ly49Q ligand on the parental BWZ cells themselves (J.R.C., unpublished observations).

To test if the reporter cell assay was functioning properly, parental BWZ.36, BWZ.Ly49Q^{BALB}, or BWZ.Ly49Q^{B6} cells were seeded in wells that had been pre-coated at various concentrations with anti-Ly49Q mAb or an isotype control mAb. BWZ.36 parental cells showed only background β-galactosidase enzyme activity after incubation with anti-Ly49Q mAb (Fig. 1*B*). In contrast, strong β-galactosidase activity was observed using BWZ.Ly49Q^{BALB} or BWZ.Ly49Q^{B6} reporter cells in response to plate-bound anti-Ly49Q mAb. Importantly, only background activity was seen for all cell lines in response to plate-bound isotype control mAb. These results demonstrate that the Ly49Q fusion receptor is folded correctly and that the BWZ.Ly49Q reporter cells are specifically stimulated by mAb-mediated receptor ligation.

Identification of Ly49Q-ligand bearing tumor cells

An unbiased screening of a panel of tumor and transformed cell lines for BWZ.Ly49Q cell stimulation was undertaken. Notably, BWZ.Ly49Q^{BALB} but not control BWZ.36 cells were stimulated with RAW, EL-4, DC2.4, C1498, and MC57G cells (Fig. 2*A*). The prevalence among the stimulating tumor cells lines of the H-2^{b} haplotype suggested that this haplotype contains a ligand for Ly49Q. Similar results were observed for the BWZ.Ly49Q^{B6} reporter cells, although the responses were significantly weaker (data not shown). This may be due to weaker receptor-ligand interactions on BWZ.Ly49Q^{B6} relative to BWZ.Ly49Q^{BALB} cells (Fig. 1*A*).

An early hypothesis was that the ligand for the relatively divergent and pDC-specific Ly49Q receptor might be a MHC-Ib, as is the case with the rat

Ly49i5 and Ly49s5 receptors (245). To test this hypothesis, a panel of cell lines expressing some of the well-characterized class Ib MHC molecules was tested for BWZ.Ly49Q^{BALB} reporter cell stimulation (Fig. 2*B*). The MHC-Ib panel tested included CD1d1, Qa1^{b}, H2-Q9, H2-Bl (also known as blastocyst MHC), and H2-M3. Although the known class Ib repertoire is much larger, none of these class Ib MHC molecules was able to stimulate the BWZ.Ly49Q^{BALB} reporter cells when expressed on the surface of various tumor cell lines (Fig. 2*B*). In contrast, BWZ.Ly49Q^{BALB} reporter cells were stimulated by RMA (H-2^{b}), but not RMA-S or B16 tumor cells (Fig. 2*B*). B16 has very low levels of surface MHC. Similarly, RMA-S is a *Tap2*-deficient subline of the RMA parental cell line that lacks surface expression of MHC Ia molecules due to an inability to transport antigenic peptides into the ER during MHC I biogenesis (251). Together, these findings indicated that Ly49Q may recognize a H-2^{b} MHC Ia-like ligand.

The Ly49Q ligand is class Ia MHC-dependent

To test whether the ligand engaging Ly49Q was also expressed on normal H-2^{b}-derived cells *ex vivo*, the reporter cell assay was used to analyze various B6-derived leukocyte populations directly, including peritoneal cells, splenocytes, bone marrow cells, thymocytes, mesenteric and popliteal lymph node cells. Strong BWZ.Ly49Q^{BALB} stimulation was observed with fresh peritoneal exudate cells (PEC), all of which were CD11b^{hi} by flow cytometry (Fig. 3*A*, left panel and data not shown). Interestingly, collagenase-treated splenocytes also weakly stimulated BWZ.Ly49Q^{BALB} cells compared to splenocytes isolated without the use of collagenase, suggesting the ligand may be expressed at higher levels on a stromal cell type.

As DCs are readily liberated from tissues by collagenase treatment, these cells were tested directly. B6-derived $CD11c^+$ splenocytes were isolated by positive selection and incubated with $BWZ.Ly49Q^{BALB}$ cells. Strikingly, a rapid and strong stimulation was observed (Fig. 3*A*, left panel). $BWZ.Ly49Q^{B6}$ were also specifically stimulated by DCs, albeit more weakly (data not shown). Furthermore, stimulation of reporter cells by DCs could be blocked by addition of anti-Ly49Q mAb, but not isotype-matched control mAb, demonstrating the receptor specificity of the stimulation (Fig. 3*A*, right panel). Thus, in addition to several H-2^b tumor cell lines, a ligand for Ly49Q is expressed on B6-derived DCs and PECs *ex vivo*. Notably, these populations are known to express high levels of both MHC and adhesion molecules, further highlighting their utility in the reporter cell assay. Therefore, DCs were used in subsequent experiments as a source of ligand-bearing cells, due to their high stimulation index and ease of isolation.

The haplotype specificity of Ly49Q recognition was next tested by isolating DC from different inbred mouse strains and testing their ability to stimulate BWZ.Ly49Q cells. DC from B6 (H-2^b), 129X1 (H-2^{bc}), and 129S1 (H-2^{bc}), but not FVB (H-2^q), BALB/c (H-2^d), A/J (H-2^a), or C3H (H-2^k) mice were able to stimulate $BWZ.Ly49Q^{BALB}$ reporter cells (Fig. 3*B*). The class Ia MHC (H-2K and H-2D) of B6 and the 129-group strains are identical, while the class Ib MHC regions have significant differences in gene content (252). To directly test whether an MHC I molecule is a ligand for Ly49Q, DC were isolated from both β_2m-deficient and TAP-1-deficient mice of the H-2^b haplotype and used in the

reporter cell assay. Neither of these MHC I-deficient DCs were able to stimulate BWZ.Ly49Q^{BALB} (Fig. 3*B*). Furthermore, a low but detectable binding of a soluble Ly49Q-Ig fusion protein to B6 bone-marrow-derived DCs was observed by flow cytometry (Fig. 3*C*). Importantly, the binding of the soluble Ly49Q protein was not observed when β_2m-deficient DCs were analyzed (Fig. 3*C*). While not all MHC I-related proteins require TAP-1/2 or β_2m for cell surface expression, these data strongly suggest that the ligand for Ly49Q is MHC I-like.

To directly test class Ia MHC molecules as candidate ligands, BWZ.Ly49Q^{BALB} cells were incubated with DC from H-2K^{b}D^b double-deficient mice. H-2K^{b}D^b-deficient DC failed to stimulate BWZ.Ly49Q^{BALB} cells, despite having mostly normal expression of class Ib MHC (Fig. 3*B*). Thus, the candidate ligand for Ly49Q is likely a MHC-Ia, and the DC-expressed ligand must either be H-2K^b, H-2D^b, or an MHC-Ib molecule dependent on MHC Ia expression. Notably, Qa1^b molecules present the Qdm leader peptide derived from the H-2D^b class Ia MHC; however, Qa1^b was formally ruled out using stably transfected cells (Fig. 2*B*).

H-2K^b is a ligand for Ly49Q

To directly test H-2K^b and H-2D^b for Ly49Q binding, soluble mouse IgG$_1$ fusions of both MHC proteins (bearing specific peptides) were used to coat wells onto which BWZ.Ly49Q cells were seeded. Following incubation, strong stimulation of both BWZ.Ly49Q^{BALB} and BWZ.Ly49Q^{B6} was detected in H-2K^b:Ig-coated wells (Fig. 4*A*). The stimulation was similar to that seen with anti-Ly49Q mAb-coated wells and only slightly less robust than that induced by

PMA/ionomycin treatment. Importantly, no stimulation was seen in wells coated with H-2D^b:Ig or control isotype Ig (mAb). Thus, H-2K^b binds directly to Ly49Q.

Other Ly49 receptors have been shown to bind H-2K^b, including Ly49C^{B6}, C^{BALB}, I^{B6}, I^{129}, and V^{129} (228, 253). Interestingly, evidence has been previously presented that Ly49C is in fact a receptor for empty or "peptide-receptive" H-2K^b (254). To test the peptide-dependence of Ly49Q recognition, the H-2K^b:Ig fusion protein was used to coat wells in the absence of added peptide. Neither BWZ.Ly49Q^{BALB} nor BWZ.Ly49Q^{B6} cells were stimulated with H-2K^b lacking peptide (Fig. 4*A*). This suggests that recognition of H-2K^b by Ly49Q is dependent on the presence of bound peptide.

As independent confirmation of Ly49Q specificity, antibody-mediated blocking of reporter cell stimulation was also performed. As expected, addition of anti-H-2K^b but not anti-H-2D^b or isotype control mAb to DC co-cultures inhibited BWZ.Ly49Q stimulation (Fig. 4*B*). Ly49Q specificity was subjected to further testing using YB2/0-derived transfectants expressing various alternate alleles. No specific stimulation was observed for YB-D^b, YB-D^d, YB-K^k, or YB-L^d (data not shown). Thus, Ly49Q appears to be H-2K^b-specific, unlike Ly49C and V, which show affinity for multiple MHC alleles in addition to H-2K^b (228, 253). Thus, H-2K^b, but not H-2D^b, is a ligand for the BALB/c and B6 alleles of Ly49Q.

In order to independently confirm the results of the BWZ assay, binding of H-2K^b to real pDCs was evaluated. Splenic pDC were isolated using anti-mPDCA1 microbeads and then stained with soluble PE-labeled H-2K^b tetramer. Significant binding of the MHC tetramer was observed by pDC (Fig. 5*A*). Contaminating lymphocytes served as an internal control and were not stained by

H-2K^b tetramer. Interestingly, pDC from K^{b}D^b-deficient mice bound higher levels of H-2K^b tetramer suggesting that, similar to other Ly49, Ly49Q interacts with its ligand in *cis*. H-2K^b tetramer is binding to Ly49Q on pDC as it can be blocked by pre-incubating pDC with anti-Ly49Q mAb but not control mAb (Fig. 5*B*). Thus, H-2K^b binding by Ly49Q is confirmed independently of the BWZ assay.

5. DISCUSSION

MHC-Ia ligands have been identified for most inhibitory or ITIM-containing members of the Ly49 family of NK cell receptors. This study presents evidence that the ligand of the divergent pDC-expressed Ly49Q protein is also class Ia MHC, specifically H-2K^b. The expression of Ly49Q on pDCs, but not NK cells, sets Ly49Q apart from the other inhibitory Ly49, but pDC and NK cells have related functional characteristics. Plasmacytoid DCs and NK cells express receptors such as TLR9 and Ly49H, respectively, that are directly capable of detecting virus infection. NK cells are also potently activated by IFN-α, a cytokine that pDCs make in abundance. Thus, the roles of pDCs and NK cells are strongly intertwined and these two cell types likely work in concert during anti-viral and perhaps other innate immune responses. The finding that both cell types are sensitive to the presence or absence of MHC supports this hypothesis.

The finding that H-2K^b is a ligand for Ly49Q was somewhat unexpected, given that fresh *ex vivo* T and B cells contained in the primary and secondary lymphoid tissue suspensions express H-2K^b, but did not significantly stimulate the BWZ.Ly49Q cells (Fig. 3*A*). One possibility for this apparent paradox is variation in the level of H-2K^b expression, as well as adhesion molecule levels, on stimulating vs. non-stimulating cell types. Flow cytometric analysis reveals that H-2K^b, but not H-2D^b, surface expression is indeed higher on B6-derived DCs (MFI=487) compared to whole splenocytes (MFI=381; unpublished observations). In addition, DCs (and PECs) possess greater levels of adhesion and costimulatory molecules than resting lymphocytes, which combined with a high

surface level of H-2K^b may overcome a threshold requirement for BWZ cell stimulation. While additional cellular signals are not required to induce BWZ stimulation via the CD3ζ fusion receptor, as supported by the cell-free stimulation using plate-bound anti-Ly49Q mAb or K^b:Ig fusion protein, these immobilized proteins induce a very strong signal. Furthermore, the stimulation of BWZ.Ly49Q by EL-4 and RMA (T cell), C1498 (leukemia), and MC57G (fibrosarcoma) tumor lines may again be facilitated by increased levels of adhesion molecules as well as greatly increased cell surface area. Thus, freshly isolated lymphocytes are smaller than DC and PEC cells, and much smaller than BWZ reporter cells and the stimulating tumor cells tested. Finally, as noted previously, the inability to achieve high level expression of the fusion-receptor and IRES-GFP cassette in BWZ.Ly49Q transductants (Fig. 1*A*) has been observed before, and may be due to co-expression on the parental BWZ cells themselves of a low-level or weak Ly49Q ligand. Thus, chronic basal stimulation (in *cis* or in *trans*) of the BWZ reporter cells in isolation can result in reporter cell death (loss of Ly49Q^{hi}/GFP^{hi} transductants), as well as an increased threshold requirement for stimulation (J.R.C., unpublished observations). The higher binding of H-2K^b tetramer by pDC of K^{b}D^b-deficient mice relative to wildtype pDC supports *cis* interactions of Ly49Q and H-2K^b (Fig. 5A).

Related to the above, our inability to detect significant BWZ.Ly49Q stimulation with other H-2K alleles and indeed other H-2 haplotypes by no means indicates that alternative, weaker ligands do not exist. In contrast, as BWZ cells are of the non-stimulating H-2^k haplotype, low endogenous levels of H-2^k or weak

Ly49Q:H-2^k interactions may actually set the threshold for detection of receptor-ligand interactions. In other words, it is possible that there is a ligand for Ly49Q^{BALB} in BALB/c mice, but that the affinity of the interaction is below the threshold of detection in this assay. This is supported by the apparent weaker stimulation of BWZ-Ly49Q^{B6} compared to BWZ-Ly49Q^{BALB} by DC and tumour cells, while both were equally stimulated by plate-bound H-2K^b:Ig (Fig. 4*A*). Alternatively, an allele of Ly49Q that recognizes BALB/c MHC may exist, but in non-BALB/c mice. Thus, the apparent absence of an MHC ligand for Ly49Q in BALB/c mice (H-2^d haplotype) may represent an artificial circumstance related to the derivation of man-made inbred mouse strains. At present, we cannot exclude these possibilities; however, with knowledge of H-2K^b as a high-affinity ligand for Ly49Q, future analysis of potential weak interactions may be facilitated using high-avidity reagents such as MHC tetramers or multimers.

Importantly, both the BALB/c and B6 alleles of Ly49Q directly recognized H-2K^b (Fig. 4*A* and 5*B*). However, BALB/c-derived DC did not stimulate Ly49Q^{BALB}-expressing BWZ cells. Therefore, notwithstanding the above, it would appear that the Ly49Q allele in BALB/c mice may either recognize a weak self ligand or have no self ligand at all. This would not be unique to Ly49Q, as this is also the case for other inhibitory Ly49 receptors. For example, Ly49A^{B6}, Ly49O^{129}, and Ly49G^{B6} can bind H-2D^d, but have no known ligands in B6 or 129 mice (228, 253). Nonetheless, this may not be a detrimental situation with respect to NK cell function, since there are usually other inhibitory receptors that recognize self MHC; however, pDCs appear to have only a single MHC-specific receptor, Ly49Q.

What is the role for an inhibitory MHC-specific receptor on pDCs? Such receptors on NK cells are hypothesized to mediate "missing-self" recognition of pathological MHC-deficient host cells during viral infections or cellular transformation. Inhibitory receptors for classical MHC have recently been suggested to also mediate the 'licensing' or 'disarming' of developing NK cells in their acquisition of self-tolerance maturation signals (255, 256). Essentially, the expression of self-MHC-specific inhibitory Ly49 receptors permits the functional maturation of NK cells that would otherwise be rendered hyporesponsive or 'anergic' due to low-level chronic engagement of stimulatory receptors such as NKG2D, NKR-P1A/C/F, or Ly49D/H. These self-tolerant mature NK cells are better able to mediate cytotoxicity and cytokine production in response to imbalances in self-nonself discrimination stimuli. By analogy, therefore, it is conceivable that Ly49Q may be responsible for similar functions in the functional maturation of pDCs. If this is the case, then why would pDCs express only a single tolerizing receptor instead of multiple inhibitory receptors like NK cells? One might hypothesize that Ly49Q might function as a pan-specific MHC receptor, like Ly49C or Ly49V, thereby obviating the need for multiple allele-specific receptors. While the data presented herein do not support this hypothesis, more sensitive methods for the detection of low affinity interactions need to be applied before making any final conclusions.

Recent reports suggesting that ITAM signaling is actually inhibitory in nature in myeloid lineage cells, including pDCs, also need to be taken into consideration when assigning a role for the ITIM-containing Ly49Q receptor. Like the co-expression of Ly49Q and H-2K^{b} on pDCs, the DAP-12-coupled

TREM2 receptor is expressed on macrophages along with an unknown ligand. The loss of either TREM2 or DAP-12 results in macrophages that are hyper-responsive to TLR stimulation and secrete higher than normal amounts of TNF-α (179). Analogous findings have been reported for pDC. Specifically, the ITAM-containing Siglec-H and DAP-12-coupled Nkp44 receptor have inhibitory effects on pDC, resulting in suppression of type I interferon secretion (33, 49). Normally, ITIM-mediated signals are dominant over ITAM-dependent pathways, yet whether the Ly49Q interaction with MHC results in suppression of stimulatory or inhibitory signals in pDCs needs to be explored. Thus, the role of Ly49Q on pDCs might be to facilitate the activation, rather than inhibition, of the innate immune system.

6. ACKNOWLEDGEMENTS

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7. FOOTNOTES

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Animal care was provided in accordance with the procedures approved by the IRCM Animal Care Committee.

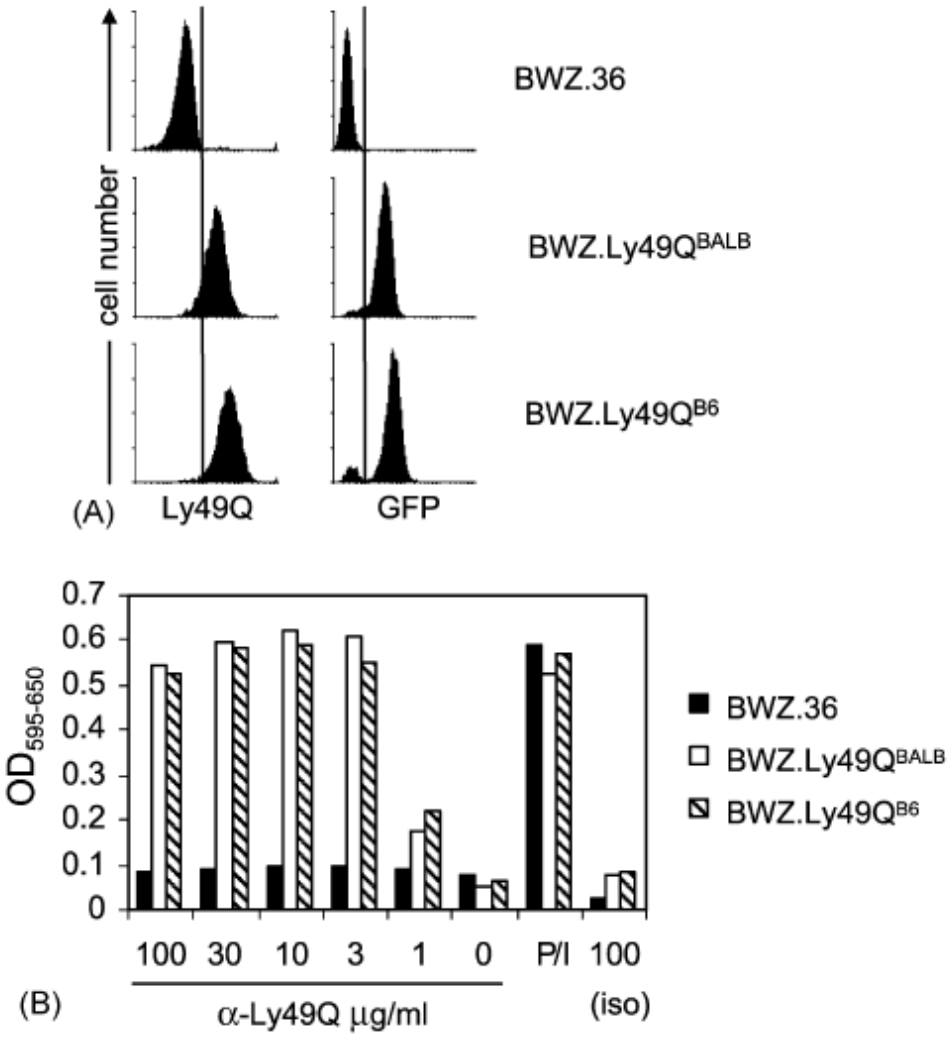

Figure 1: Ly49Q-CD3ζ fusion receptor-bearing BWZ cells are stimulated by Ly49Q cross-linking. (A) BWZ.36 parental cells or transductants expressing a chimeric protein (introduced by a retrovirus containing IRES-GFP) with the extracellular domain of either B6 or BALB/c Ly49Q proteins were stained with anti-Ly49Q mAb and analyzed by flow cytometry. Both anti-Ly49Q (NS-34) staining (left column) and GFP expression (right column) are shown for parental and transduced cells. (B) BWZ.36 parental cells and Ly49Q-CD3ζ transductants were seeded in wells pre-coated with various concentrations of anti-Ly49Q (2E6) mAb or isotype (iso) control antibodies. After overnight incubation, lacZ production was revealed by addition of CPRG substrate and optical density measurements at the indicated wavelengths. LacZ production capability by BWZ cells is shown by PMA/ionomycin (P/I) treatment.

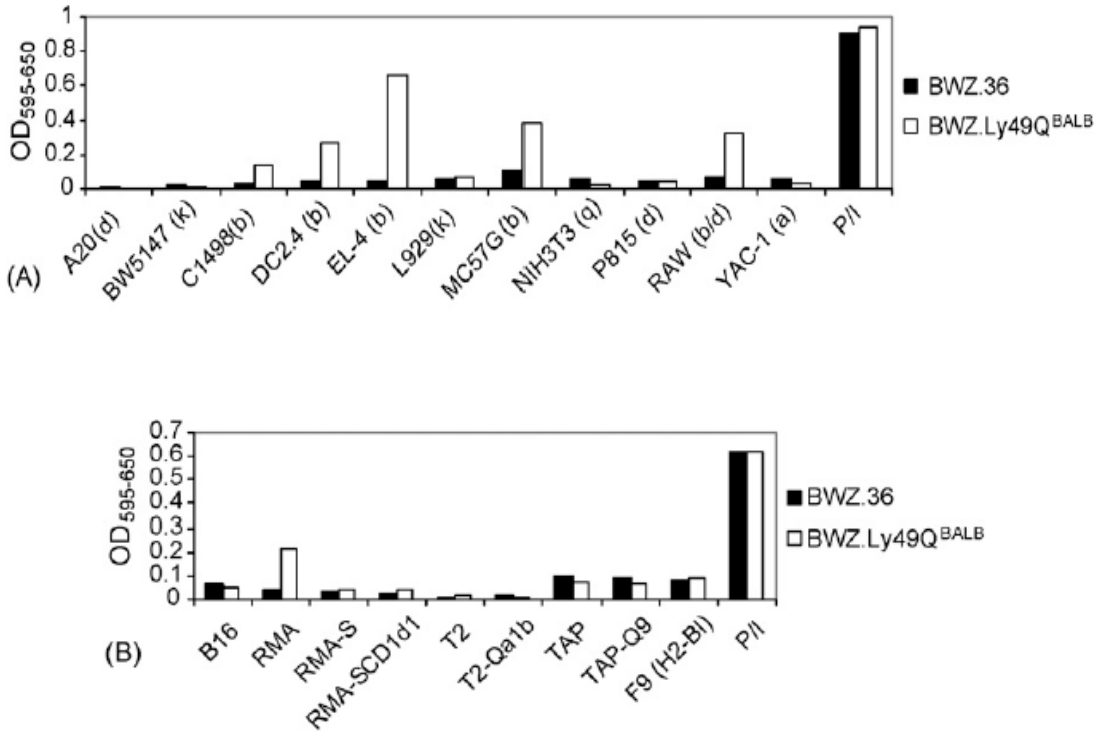

Figure 2: H-2^{b} tumor cells stimulate BWZ.Ly49Q reporter cells. BWZ.36 parental or BWZ.Ly49Q^{BALB} cells were co-incubated with various indicated (A) tumor lines and (B) class Ib MHC-transfected sublines at a 1:1 ratio overnight followed by addition of CPRG substrate. The H-2 haplotype is indicated in parentheses in (A). Data representative of three independent experiments is shown.

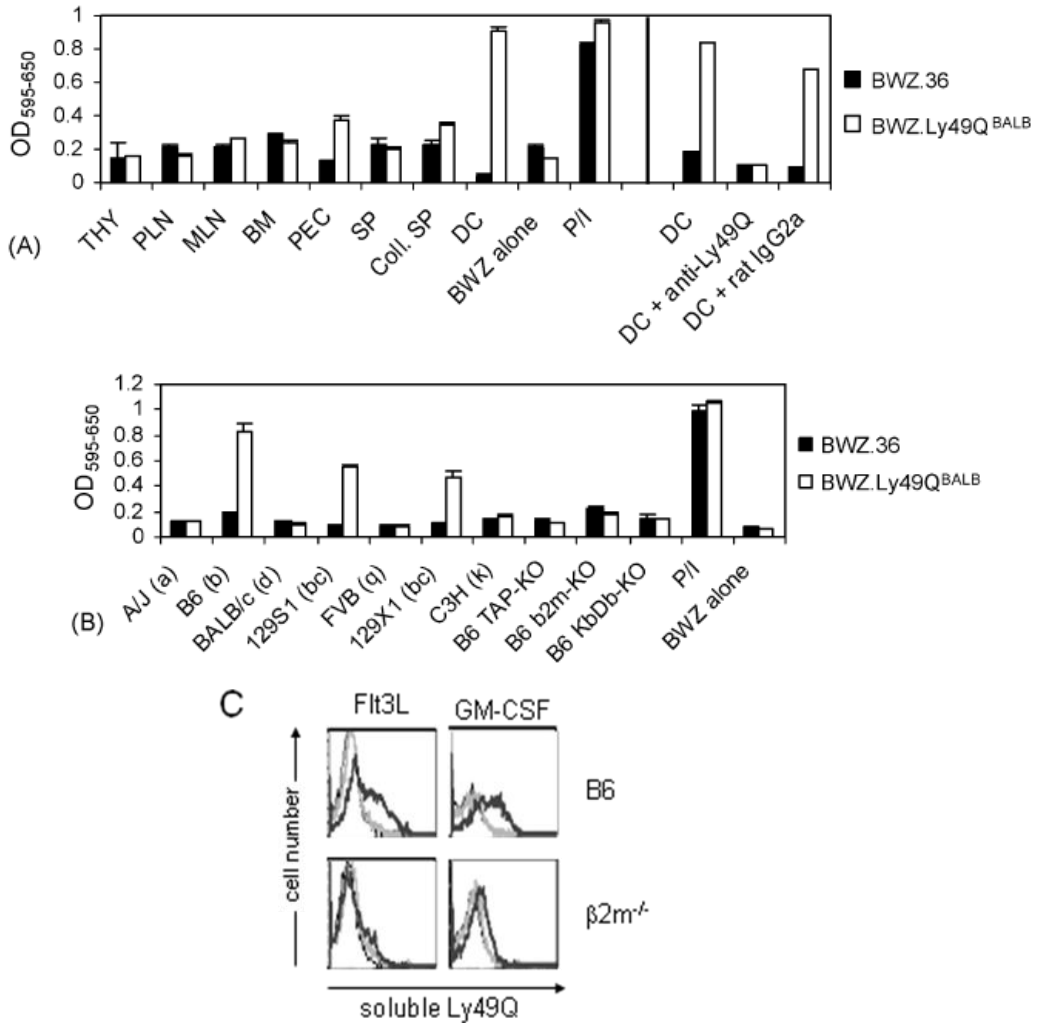

Figure 3: H-2^b-derived DCs stimulate BWZ.Ly49Q in a class Ia MHC-dependent manner. (A) Single cell suspensions were prepared from the indicated tissues of B6 mice and incubated with BWZ.Ly49Q^{BALB} reporter cells followed by addition of CPRG substrate (left panel). DCs were isolated using anti-CD11c microbeads and incubated with BWZ cells in the presence of 2E6 (anti-Ly49Q) or isotype-matched control mAb (right panel). THY, thymus; BM, bone marrow; SP, spleen; PEC, peritoneal exudate cells; MLN, mesenteric lymph nodes; PLN, popliteal lymph nodes; Coll. SP, collagenase-treated spleen. (B) $CD11c^+$ DCs isolated from the indicated inbred and gene-knockout mouse strains were co-incubated with BWZ reporter cells overnight followed by addition of CPRG substrate. The H-2 haplotype of the inbred strains is indicated in parentheses. (C) Flt3L-cultured (left column) or GM-CSF-cultured (right column) bone-marrow DCs from B6 or $\beta_2m^{-/-}$ mice were treated with IFN-α and then stained with a soluble Ly49Q-Ig fusion protein. Surface binding of $CD11b^+CD11c^+$ gated cells was analyzed by flow cytometry and are shown as histograms. Thick black line, soluble Ly49Q; thick gray line, human IgG (control); thin black line, secondary antibody alone. Data representative of three independent experiments is shown.

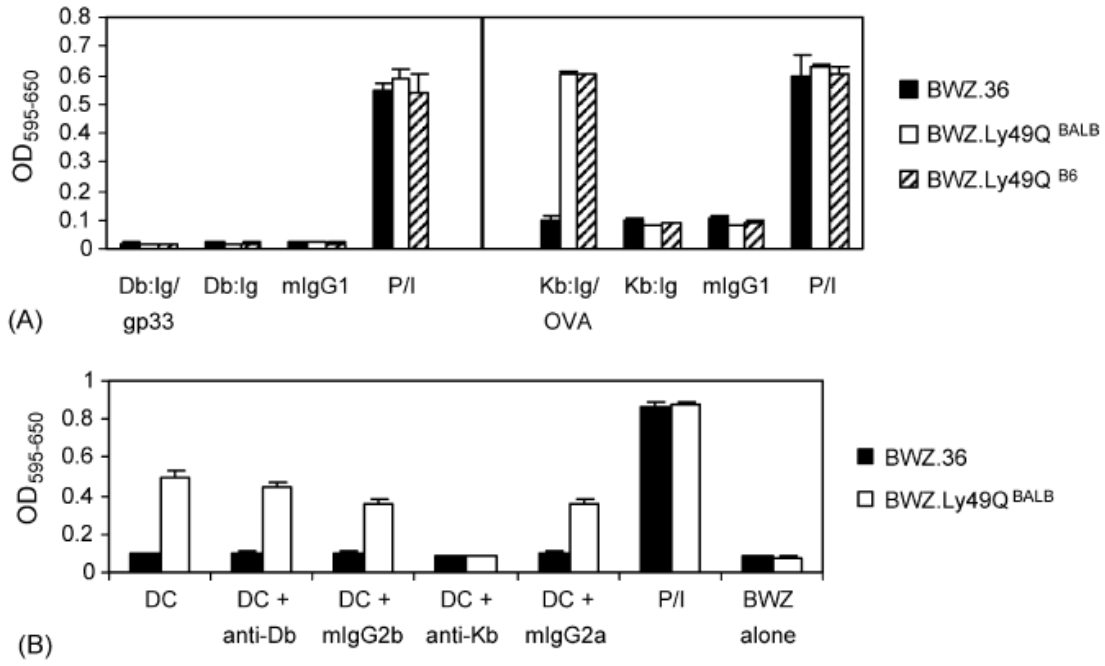

Figure 4: H-2K^b, but not H-2D^b, is a ligand for Ly49Q. (A) BWZ.36 parental, BWZ.Ly49Q^{B6}, and BWZ.Ly49Q^{BALB} reporter cells were seeded onto wells that had been pre-coated with peptide-bearing or peptide-receptive H-2K^b:Ig or H-2D^b:Ig fusion proteins, or wells pre-coated with control mAb matched to the isotype of the MHC fusion protein (mouse IgG_1). (B) BWZ reporter cells were incubated with B6-derived DC in the presence of blocking anti-H-2K^b, anti-H-2D^b, or control mAbs. Analysis was performed as described in Figure 1. Data representative of three independent experiments is shown.

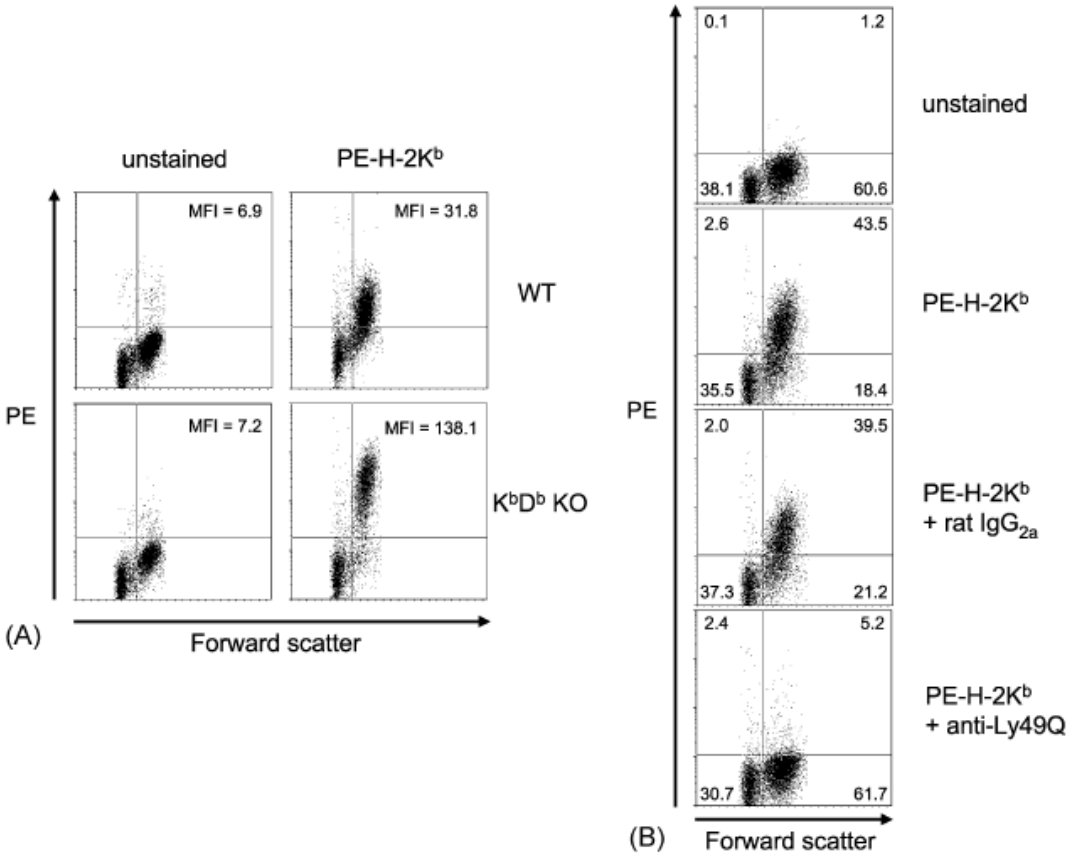

Figure 5: Soluble H-2K^b binding to pDC is Ly49Q-dependent. (A) Splenic pDCs were isolated using anti-mPDCA1 microbeads from wildtype or K^{b}D^b-deficient mice and stained with soluble PE-H-2K^b tetramer bearing OVA peptide. Cells were gated to include pDC and lymphocytes. The MFI of pDCs only is indicated. (B) Splenic pDCs from K^{b}D^b-deficient mice were pre-incubated with anti-Ly49Q mAb or isotype control and then stained with PE-H-2K^b/OVA tetramer. Cells were gated to include pDCs and lymphocytes. The percentage of cells in each quadrant is indicated.

PREFACE TO CHAPTER 3

Since we identified the MHC class I molecule H-2K^b as the Ly49Q ligand (257), several subsequent reports have confirmed our findings (224, 258). Identification of H-2K^b as the ligand for pDC suggests that similar to NK and T cells, pDC function appears to be regulated by MHC class I molecules. Having successfully identified the Ly49Q ligand, we proceeded with a more targeted approach to characterizing the function of the Ly49Q:H-2K^b interaction.

Despite significant structural differences between the mouse Ly49 and human KIR receptors, their functions are conserved and many important advances in murine innate immune cell research have been directly applicable to humans. In this view, a total Ly49 cluster deletion has been generated and is currently being studied in our laboratory. This mouse will be an invaluable resource to understand the role of NK cells in immune responses and how this is regulated by the family of Ly49 receptors. As a part of this greater project of completely deleting the Ly49 cluster, our laboratory has generated a mouse with a targeted disruption of the *Ly49q$_1$* gene, which allows us to study the individual contribution of this pDC receptor to innate immunity.

Armed with both the identity of the Ly49Q ligand and a Ly49Q-deficient mouse, we can proceed to dissect the role of mouse pDC receptor Ly49Q.

CHAPTER 3

Positive Regulation of Plasmacytoid Dendritic Cell Function via Ly49Q Recognition of Class I MHC

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Total character counts (not including spaces): 30,871

Running Title: Ly49Q Regulation of pDC Function

1. ABSTRACT

Plasmacytoid dendritic cells (pDCs) are an important source of type I IFN during initial immune responses to viral infections. In mice, pDCs are uniquely characterized by high-level expression of Ly49Q, a C-type lectin-like receptor specific for MHC I molecules. Despite having a cytoplasmic ITIM, Ly49Q was found to enhance pDC function *in vitro*, as pDC cytokine production in response to the TLR-9 agonist, CpG-ODN, could be blocked using soluble mAb to Ly49Q or H-2K^b. Conversely, CpG-ODN-dependent IFN-α production by pDCs was greatly augmented upon receptor cross-linking using immobilized anti-Ly49Q mAb or recombinant H-2K^b ligand. Accordingly, Ly49Q-deficient pDCs displayed a severely reduced capacity to produce cytokines in response to TLR-7 and TLR-9 stimulation both *in vitro* and *in vivo*. Finally, TLR-9-dependent anti-viral responses were compromised in Ly49Q-null mice infected with MCMV. Thus, MHC I recognition by Ly49Q on pDCs is necessary for optimal activation of innate immune responses *in vivo*.

2. INTRODUCTION

Plasmacytoid dendritic cells (pDCs) are potent anti-viral effector cells that were originally identified by their plasma cell-like morphology and localization within the T cell zone of lymphoid tissue (241). Also termed type I interferon producing cells (IPC), pDCs secrete more type I interferon on a per-cell basis than any other cell type (4, 259, 260). Plasmacytoid DCs are especially important in controlling viral infections, a property highlighted by their selective expression of TLR-7 and TLR-9 (108), which recognize single-stranded (ss)RNA and double-stranded (ds)DNA, respectively. Plasmacytoid DCs do not express TLR-2, TLR-3, TLR-4 and TLR5, explaining why they do not respond to common bacterial products recognized by other APCs.

Plasmacytoid DCs represent a rare cell type constituting about 1% of bone marrow or splenic leukocytes and less than 0.5% of lymph node and peripheral blood leukocytes. However, their frequency varies between mouse strains with 129Sv mice possessing a significantly higher proportion of pDC than other mouse strains (32). Mouse pDC do not express the lineage markers CD19, CD3, DX5, CD14 or TER119 (13, 14). In addition to their selective pattern of TLR expression, pDC and myeloid dendritic cells (mDCs) are dissimilar in various other aspects. Unlike mDCs, pDCs are characterized by a $CD11b^{-}B220^{+}Ly6C^{+}$ phenotype (13). Like myeloid dendritic cells (mDC), pDCs express CD11c, but they do so at a lower level (8). Resting pDCs have been referred to as immature APCs since they express only low levels of CD86 and class II MHC, and they display little or no endocytic activity. However, upon TLR stimulation all three of these characteristics are upregulated to allow pDCs to present antigenic peptides

and optimally stimulate $CD4^+$ T cell function (13). In addition, pDCs have been implicated in promoting mDC maturation and terminal B cell differentiation to functional antibody-producing plasma cells (241). Five different mAb reagents have been reported to specifically recognize murine pDC: 120G8 (32), mPDCA-1, 440c (242), NS-34 (35) and 2E6 (243). The 440c mAb recognizes Siglec-H, a DAP-12 coupled receptor that inhibits pDC function, including IFN-α secretion (33). 120G8 and mPDCA-1 both recognize bone marrow stromal cell antigen 2 (BST-2) (31). NS-34 and 2E6, recognize Ly49Q, a member of the type II C-type lectin-like Ly49 family. Interestingly, most other Ly49 family member are exclusively expressed on NK, NKT, and T cell subsets, where they are known to regulate cytokine production and cell-mediated cytotoxicity via interactions with cognate MHC class I-related ligands on target cells.

Ly49Q is one of the more distantly related Ly49 family members, yet the receptor itself is highly conserved among three mouse haplotypes (B6, 129S6, and BALB/c) (236, 237, 249). To date, Ly49Q protein has been detected in all mouse strains tested (238), suggesting an important and conserved function for this receptor. The *Ly49q* gene defines the centromeric end of the B6, 129S6, and BALB/c *Ly49* gene clusters. Interestingly, a homologous segment comprising *Ly49q*- and *Ly49e*-like genes is repeated three times in the 129S6 genome due to gene duplication (240). Therefore, in addition to $Ly49q_1$, the 129-related mouse strains contain $Ly49q_2$ and $Ly49q_3$, but the latter two genes are considered pseudogenes because they lack exons 6 and 7, which encode the ligand-binding domain (240).

Ly49Q was first reported to be expressed at low levels on a proportion of Gr-1^{+} bone marrow myeloid precursor cells, on peripheral blood neutrophils (Gr1^{+}CD11b^{+}), and on IFN-γ-activated macrophages (35). However, the function of the receptor on these cell types remains unknown. Ly49Q contains a cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM), while it lacks a positively-charged transmembrane residue, both of which are characteristics of inhibitory Ly49 receptors expressed by NK cells. Furthermore, like inhibitory Ly49 NK receptors the Ly49Q ITIM has been reported to associate with the SHP-1 and SHP-2 phosphatases upon antibody-mediated cross-linking of the receptor (35). However, Ly49Q is not expressed by NK cells. Moreover, Ly49Q cross-linking on activated macrophages has been reported to induce cytoskeletal rearrangement leading to formation of polarized filopodia and lamellopodia; this suggests a role for Ly49Q in macrophage migration and phagocytosis (35).

In subsequent reports a population of cells expressing significantly higher levels of Ly49Q than neutrophils and macrophages was identified (111, 238, 243). This cell population was originally defined as CD11c^{+}B220^{+}Gr1^{low} and has been confirmed to represent pDCs (43, 241). Virtually all peripheral pDCs and the majority of bone marrow pDCs express Ly49Q. The subset of bone marrow pDCs lacking Ly49Q expression are thought to represent immature cells, such that acquisition of Ly49Q expression is linked to sequential development of functional pDC (111, 243). These Ly49Q^{-} pDCs do not respond to certain stimuli and are defective in secretion of some cytokines. Ly49Q levels correlate well with pDC

maturation and receptor acquisition is further upregulated by various stimuli including IFN-α (111, 238). In some but not all inbred mouse strains a subset of mDC also express low levels of Ly49Q (238).

We recently identified the classical MHC class I molecule, H-2K^b, as the cognate Ly49Q ligand (257). Using reporter cell analysis, a high-affinity ligand for Ly49Q was detected on tumor and normal *ex vivo* cells derived from H-2^b haplotype mice (C57BL/6, 129), but not on cells from other mouse MHC haplotypes tested. Direct MHC surveillance by pDCs and the implications of this interaction for innate immunity remain to be investigated.

The current study demonstrates a major role for Ly49Q/H-2K^b interactions in pDC production of IFN-α and, to a lesser extent, IL-12. Remarkably, the function of Ly49Q on pDCs appears to be stimulatory in nature as revealed by cross-linking experiments despite the presence of a cytoplasmic ITIM motif. To confirm these *in vitro* findings and to ascertain the role of Ly49Q during immune responses, Ly49Q-null mice were generated and characterized. Ly49Q-null mice exhibit a severe defect in systemic IFN-α production after challenge with agonists and pathogens recognized by TLR-7 and TLR-9, which translates into weaker anti-viral responses *in vivo*. We propose that Ly49Q recognition of self MHC is necessary to regulate pDC cytokine responses to pathogens.

3. MATERIALS AND METHODS

Mice

129S1 and C57BL/6 mice were purchased from The Jackson Laboratory. *H-2Kb^{tm1}H-2Db^{tm1}* (K^bD^b-KO) mice on a B6 background were purchased from Taconic (Hudson, NY). Mice were maintained and bred in the IRCM specific-pathogen free animal facilities. All manipulations performed on animals were in accordance with IRCM Institutional guidelines and approved by the IRCM Animal Ethics Committee. Unless otherwise mentioned all mice used for experiments were 5-8 weeks of age.

Generation of Ly49Q-null Mice

We previously identified 129S6 BAC clones containing *Ly49q$_1$* (15). A 10 kb segment centered on exon 2 was retrieved from BAC 34o6 into pMCITKpANhe vector by lambda red-mediated recombineering in EL350 cells as previously described (261). To complete the targeting construct, a floxed PGK-neomycin cassette from PL400 was inserted into a unique StuI site in exon 2. The complete targeting construct sequence was confirmed. R1 ES cells (129Sv x 129Sv-CP) were electroporated with NotI-linearized targeting vector DNA and then selected in G418. Homologous recombination in ES cells was assessed by Southern blot. A homologous recombination efficiency of 1% was observed (6/600). The germline competency of these clones was confirmed by generating heterozygous founder mice, which were then bred to achieve Ly49Q$^{neo/neo}$ homozygosity. An ES clone carrying a properly targeted *Ly49q$_1$*^{neo} allele was electroporated with a CMV-Cre vector (a kind gift from Dr. David Lohnes, University of Ottawa) and screened for neomycin deletion by PCR. *Ly49q$_1$*^{neo} and *Ly49q$_1$*^{lox} founder mice

were generated by the IRCM MicroInjection Service. Founder mice that could transmit the targeted *Ly49q*$_1$ allele to the germline were bred to 129S1 females to maintain a 129-background, which was confirmed for the *Ly49* locus by 4E5 vs. 12A8 staining of NK cells as previously described (253). Resulting heterozygous mice were bred and homozygous WT and Ly49Q-null littermates were used in all experiments unless otherwise indicated. Southern blot analysis for the *Ly49q*^{neo} allele was performed using an intron 4 probe cloned into pCR2.1TOPO (Invitrogen) prepared by amplifying BAC 34o6 DNA with: sense primer, 5'-TAT GAC TTC TTG GAG AGA GT-3'; antisense primer, 5'-TTC ACG TGG GCC TAG AAT TT-3'. The *Ly49q*$_1^{lox}$ allele was detected by PCR with: sense primer, 5'-CCT AAA AGT AAT TGC TGT GAC TAT T-3'; antisense primer, 5'-CTT TCT AAC TAG CTA ACA ACA G-3'. A substitute reverse primer was used to amplify the *Ly49q*$_1^{neo}$ allele: 5'-CCG AAT ATC ATG GTG GAA AAT GGC-3'. PCR cycling parameters used were: 94°C 30s, 55.5°C 30s, 72°C 30 s, 35 cycles.

pDC isolation and in vitro activation

Mouse spleens were injected with collagenase (Roche Diagnostics), minced, and then incubated for 20 min at 37°C. Following incubation, splenocytes were crushed in cold PBS-BSA-EDTA and strained through a 70 μM filter. RBC were removed by incubation with ACK lysis buffer for 5 min at 4°C. Isolated splenocytes were maintained throughout the procedure in cold PBS-BSA-EDTA. Anti-mPDCA-1 conjugated microbead (Miltenyi Biotec) labeling was then performed according to the manufacturer's protocol. Isolated pDC were seeded at 100,000 cells per well in 200 μl of pDC medium (RPMI, 10% FBS, pen/strep, L-

glutamine, 2-β-mercaptoethanol) and cultured for 16 h at 37°C/5% CO_2 in a humidified atmosphere in the presence of CpG-ODN 10103 or 2336 (Coley Pharmaceuticals, Ottawa, ON), imiquimod, MCMV or influenza at the indicated concentrations/PFU/HAU. Supernatants were collected and frozen.

In vivo pDC activation

Mice were injected through the tail vein with CpG-ODN plus 1,2-dioleoyloxy-3-trimethylammonium-propane (DOTAP; Roche) preparation (170μl CpG-ODN diluted in sterile PBS plus 30μl of DOTAP) or imiquimod (R-837; Invivogen, San Diego, CA) in 200 μl of PBS. Mice were bled through the cheek vein at indicated time points after injection. Serum was analyzed by ELISA.

Virus infections

Influenza strain A HK X31 H3N2 was a kind gift of Dr. Tania Watts (University of Toronto, Toronto, ON). MCMV (Smith strain) was a kind gift of Dr. Silvia Vidal (McGill University, Montreal, QC). MCMV preparation, injection, and plaque assay was performed as previously described (262).

ELISAs

Mouse IFN-α was determined by sandwich ELISA. Briefly, 96-well microtiter plates were coated overnight at 4°C with 5μg/ml of rat anti-mouse IFN-α antibody (RMMA-1) (PBL Biomedical Laboratories) diluted in 0.1M sodium carbonate buffer. Wells were blocked for 1h with PBS/10% FBS and then incubated for 2h with samples or a recombinant mouse IFN-α standard (Sigma). IFN-α was detected with 100 ng/ml of polyclonal rabbit anti-mouse IFN-α antibody (PBL Biomedical Laboratories) for 24h at room temperature, washed,

incubated for 1h with a donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham), followed by 20 min in TMB substrate (Sigma) and 1M H_2SO_4 was added to stop the reaction. Between incubations, plates were washed three times with PBS/0.05% Tween-20. Optical density was read at 450nm with a microtiter plate reader (Molecular Device). IL-12p70, IL-6, and TNF-α production by pDC was assayed using commercial ELISA kits (BD Biosciences).

Immunofluorescence and FACS Analysis

The following FACs mAb were used: biotin-440c (anti-Siglec-H; Cell Sciences, Canton, MA), APC-mPDCA-1 (anti-BST2; Myltenyi Biotec, Auburn, CA), FITC-CD86, 2E6 (anti-Ly49Q; eBioscience), APC-B220, PE-CD11c, and SA-PE were purchased from BD Pharmingen. NS-34 (anti-Ly49Q) was prepared as previously described (10). PE-conjugated H-$2K^b$/OVA tetramer contains human β2m and was purchased from Beckman Coulter (Fullerton, CA). For all FACS analyses, cells were first washed in FACS buffer (PBS, 0.5% BSA, 0.02% NaN_3), then incubated for 20 min at 4°C with fluorochrome conjugated antibodies. Two-step staining involved an additional incubation for 20 min with the appropriate secondary antibody at 4°C followed by a wash in FACS buffer. Stained cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences). Three-color immunofluorescent staining with biotin-rat anti-mPDCA-1 plus SA-Alexa 594, hamster anti-CD3ε (BD Pharmingen) plus Alexa 647-goat anti-hamster IgG (Invitrogen), and FITC-anti-CD19 (BD Pharmingen) was performed on slide-mounted 10μm spleen sections from untreated and CpG-ODN-injected mice. Slides were viewed on a Zeiss Confocal Axiovert 100M.

Giemsa staining

Fresh or CpG-ODN-cultured pDCs were cytocentrifuged onto glass slides, fixed in methanol for 10 min, and air-dried. Slides were stained with modified Giemsa Stain Solution (Sigma-Aldrich, Toronto, ON) diluted 1/10 in dH_2O for 10 min, rinsed with dH_2O and mounted. Pictures were obtained with Zeiss Axiophot.

Statistical analysis

Unless otherwise indicated, all P values were calculated with the two-tailed Student's *t*-test.

4. RESULTS

Ly49Q interactions with class I MHC are necessary for IFN-α secretion by pDCs

We have previously shown that H-2K^b is a ligand for Ly49Q (257), suggesting that pDC function may be regulated by class I MHC. Therefore, we investigated the influence of Ly49Q:MHC I interactions on type I IFN secretion by pDCs in response to innate immune stimuli. As previously reported, overnight culture of splenic pDCs (129S1 strain) in the presence of CpG-ODN resulted in robust IFN-α production (32). However, when blocking mAb (257) specific for either Ly49Q or H-2K^b were added at the initiation of cell cultures IFN-α secretion by pDCs was almost completely abrogated (Fig. 1 A). IL-12 secretion by pDCs could also be blocked using Ly49Q or H-2K^b mAb (Fig. 1 B). Thus, interaction of the ITIM-containing Ly49Q receptor with cognate H-2K^b ligand appears to be necessary for efficient TLR9-induced cytokine production by pDCs.

To test the possibility that Ly49Q is directly involved in activating pDCs and stimulating IFN-α production upon receptor ligation, plate-bound mAb-mediated crosslinking experiments were employed. Since pDCs from B6 mice are low producers of type I IFN compared to 129 mice (32), B6 pDCs were selected to enhance detection of increased IFN secretion. As previously reported B6 pDCs cultured in the presence of CpG-ODN produced low levels of IFN-α. In contrast, when B6 pDCs were cultured on immobilized Ly49Q mAb, IFN-α production was increased 10-15 fold (Fig. 1 C). IFN-α secretion was also induced using immobilized recombinant ligand (K^b:Ig/OVA) showing MHC directly activates

pDCs. When this experiment was repeated with 129S1-derived pDC, anti-Ly49Q cross-linking also significantly increased IFN-α production in response to CpG-ODN, albeit to a lesser extent (Fig. 1 E). Collectively, these results suggest that pDC are synergistically activated by MHC-I and TLR-9 ligands.

The activation of pDCs through Ly49Q, an ITIM-bearing receptor, contrasts with the established functions of similar receptors in lymphocytes. However, our findings are complementary to those of recent studies reporting that ITAM-coupled receptors inhibit pDC activation, specifically IFN-α production (33, 49). To determine the outcome of simultaneous ITIM- and ITAM-mediated signal transduction in pDCs, purified cells were cultured in the presence of CpG-ODN on immobilized anti-Ly49Q, anti-Siglec-H (a DAP12-coupled receptor), or both. Using both B6- and 129-derived pDCs, inhibitory Siglec-H crosslinking blocked Ly49Q activation and suppressed IFN-α secretion (Fig. 1 D,E). Notably, the reported inhibitory effect of anti-Siglec-H was only seen with 129-derived pDCs, likely due to the larger basal IFN-α response seen using 129- versus B6-strain pDC. Thus, ITAM-mediated inhibitory signals appear to be dominant and suppress ITIM-mediated stimulatory signals in pDCs.

Targeted disruption of the Ly49q$_1$ gene

To confirm the role of Ly49Q in pDC function, we sought to determine the effect of Ly49Q deficiency by targeted gene disruption *in vivo*. The *Ly49q$_1$* gene was disrupted in 129-background ES cells by inserting a floxed neomycin cassette into the StuI site of exon 2 adjacent to the ITIM coding region (Fig. 2 A). Founder mice shown to carry the *Ly49q$_1^{lox}$* allele and to exhibit germline

competence were bred to 129S1 females. The resulting heterozygous offspring were interbred and genotyped by PCR or Southern blotting (Fig. 2 B-D). Note that the *Ly49q*$_1$ probe also detects the closely related *Ly49q*$_2$ and *q*$_3$ pseudogenes of the 129 gene cluster, as all three genes display the same WT KpnI fragment (Fig. 2 B). However, mice heterozygous or homozygous for the *Ly49q*$_1^{neo}$ or *Ly49q*$_1^{lox}$ alleles are easily distinguished by PCR using *Ly49q*$_1$-specific primers that do not detect the *Ly49q*$_2$ or *q*$_3$ pseudogenes (Fig. 2 C,D).

The insertion of a *PGK-neo* cassette or *loxP* site into exon 2 did not eliminate transcription as revealed by RT-PCR (data not shown). Analysis of the targeted cDNA revealed that insertion of *PGK-neo* or *loxP* interrupted the open reading frame, leading to premature termination. Both pDCs and mDCs from *Ly49q*$_1^{neo/neo}$ and *Ly49q*$_1^{lox/lox}$ mice lack any detectable Ly49Q by flow cytometry (Fig. 2 E and data not shown). We have reported that, in addition to pDCs, neutrophils also express low levels of Ly49Q protein (35). *Ly49q*$_1^{lox/lox}$ blood neutrophils lacked all detectable reactivity with anti-Ly49Q mAb (data not shown), confirming that Ly49Q is normally expressed at low levels on this cell type. Collectively, these data demonstrate that the open reading frame of *Ly49q*$_1$ was disrupted successfully.

The effect of introducing *PGK-neo* or a *loxP* site in *Ly49q*$_1$ on neighboring gene expression was assessed by flow cytometry on both resting and IL-2 cultured NK cells with mAb recognizing proteins encoded by genes 5' (*Ly49g*, *Ly49r*, and *Ly49e/s*) and 3' (*Nkg2a/c/e* and *Nkg2d*) of *Ly49q*$_1$. Both the distribution and levels (MFI) of these receptors were the same on *Ly49q*$^{wt/wt}$, *Ly49q*$^{neo/neo}$, and *Ly49q*$^{lox/lox}$ NK cells (data not shown). This indicates that the targeted insertion had no

secondary effect on other genes located within the NKC. Furthermore, Ly49Q-null pDCs H-2K^b and H-2D^b surface levels were identical to those of Ly49Q-WT pDCs (data not shown). As *Ly49q*$_2$ and *q*$_3$ are likely pseudogenes, *Ly49q*$_1$ will be referred to as *Ly49q* hereafter. Unless otherwise stated, all experiments were performed with *Ly49q*$^{wt/wt}$ (WT) or *Ly49q*$^{lox/lox}$ (Ly49Q-null) littermates in the high IFN-producing 129 background.

Hematopoiesis and pDC development in Ly49Q-null mice

Fresh splenic pDC are round cells with eccentric kidney-shaped nuclei as visualized by Giemsa stain (Fig. 3 A). After activation with CpG-ODN, pDCs were larger, displayed a higher cytoplasmic to nucleus content, and the cytoplasm also appeared more vacuolated. The size and morphology of resting or activated pDCs from Ly49Q-WT and null mice were similar. To determine whether the localization of Ly49Q-null pDCs is normal, three-color immunofluorescent staining using mPDCA-1, CD3, and CD19 mAb was performed on splenic sections from untreated and CpG-ODN-treated animals. In the spleens of untreated animals, pDCs were mainly localized within the T cell zones and in lower numbers within marginal zone and red pulp (Fig. 3 B). In CpG-ODN treated animals, the number of pDCs was increased and they tended to form clusters (Fig. 3 B). The majority of CpG-activated pDCs were found within the marginal zone and red pulp as previously reported (58). Thus, no significant differences were observed in the localization of pDCs in WT and Ly49Q-null mice before or after CpG-ODN treatment.

The potential effect of Ly49Q absence on pDC frequency and distribution of cells in primary and secondary lymphoid organs was investigated in the

Ly49Q-null mice. The pDC population as defined by $CD11c^+Siglec\text{-}H^+$ showed no significant difference in the proportion and total number of pDCs in the spleen, mesenteric lymph nodes, or bone marrow of young (6 week-old) mice (Fig. 3 C and data not shown). However, in older mice (>9 month-old) there was approximately a two-fold increase in the proportion and total number of pDCs in these organs (Fig. 3 C, and data not shown). Myeloid lineage cells in various organs have been reported to express Ly49Q. There were no significant differences in the percentage and total number of $CD11b^+Gr\text{-}1^+$ cells in the bone marrow, spleen, or lymph nodes in either young or old mice (data not shown). Similarly, blood neutrophil numbers and proportion in WT mice and Ly49Q-null littermates were not significantly different (data not shown). In fact, no significant difference for any lineage tested was observed including the B, NK, NKT, $CD4^+$ T, $CD8^+$ T cells, or mDC subsets in either young or old mice (data not shown). Therefore, Ly49Q-deficiency appears to selectively impact pDC development and homeostasis, albeit only modestly. Young mice (5-8 weeks) were chosen for all functional experiments.

Severe defect in TLR-9-induced IFN-α production by Ly49Q-null pDCs

To determine the effect of Ly49Q-deficiency on pDC activation and subsequent cytokine production, the response of Ly49Q-null pDCs to TLR-9 agonists was evaluated. Splenic pDCs were isolated from WT and Ly49Q-null littermates using mPDCA-1-conjugated magnetic beads, cultured overnight in various concentrations of CpG-ODN, and the supernatant was assayed for IFN-α. Remarkably, Ly49Q-null pDCs supernatants yielded approximately 5-fold lower

levels of IFN-α relative to supernatants from identically-treated WT pDCs (Fig. 4 A). This finding is consistent with the earlier observation that Ly49Q binding to H-2K^b is necessary for TLR9-mediated IFN-α secretion (Fig. 1 A). In parallel, Ly49Q-deficient pDCs showed a significant defect in IL-12 secretion in response to CpG-ODN compared to WT pDCs (Fig. 4 B). The IL-12 defect was not as pronounced as that observed for IFN-α, but is consistent with the decrease in IL-12 production observed upon mAb-mediated blockade of Ly49Q-H-2K^b interactions (Fig. 1 B). In contrast, TNF-α and IL-6 production was not compromised in Ly49Q-deficient pDC (Fig. 4 C and D). Thus, Ly49Q-deficient pDCs possess an intrinsic defect in IFN-α production following stimulation with TLR9 agonists *in vitro*.

Injection of mice with CpG-ODN leads to a rapid elevation of serum IFN-α peaking at six hours post-treatment that is mediated by pDC (58). To determine whether the *in vitro* cytokine defect of Ly49Q-null pDCs had *in vivo* consequences, mice were injected with CpG-ODN, then serum IFN-α levels were evaluated after six hours. Importantly, IFN-α serum levels in CpG-injected Ly49Q-null mice were consistently 5-10 fold less than those of WT littermates (Fig. 4 E). On the other hand, the sera of both cohorts showed similar levels of IL-12 (data not shown). The normal serum IL-12 levels detected in Ly49Q-null mice following CpG injection were most likely due to TLR-9-mediated IL-12 production by cell types other than pDCs (263). A time-course experiment monitoring serum IFN-α levels confirmed that while maximal IFN-α levels occur 4-6 hours post injection in WT mice, Ly49Q-null mice failed to attain a similar

magnitude of IFN-α levels at any time point assayed (Fig. 4 F). Thus, Ly49Q-null mice display an inherent defect in IFN-α production *in vivo*, and IFN-α and IL-12 responses to CpG-ODN can be uncoupled in these mice. While the absence of the receptor leads to clear defects in cytokine production the effect of loss of the ligand is unknown. Thus, pDCs were isolated from H-2K^{b}D^b-null mice and stimulated for IFN-α production by CpG-ODN. H-2K^{b}D^b-null pDCs showed ~50% reduction in IFN-α secretion compared to WT pDCs (Fig. 4 G). These results are consistent with the hypothesis that Ly49Q:MHC interactions are necessary for cytokine production in pDCs, although the inhibition of IFN-α secretion was not as pronounced as for Ly49Q-null pDCs. Possible reasons for this difference may include mouse-strain background or that Ly49Q may have other ligands in addition to H-2K^b.

To address the question of whether Ly49Q:MHC interact in *cis* or *trans* to regulate pDC function, Ly49Q-null mice were crossed to H-2K^{b}D^b-null mice and the resulting F1 mice were intercrossed to generate the four possible null phenotypes. Plasmacytoid DCs from WT mice stain positively with soluble H-2K^b/OVA tetramer, this binding is due to Ly49Q as shown by the complete absence of binding in Ly49Q-null mice and by previous blocking experiments with anti-Ly49Q mAb (Fig. 5 A, compare top two dot plots). In the absence of H-2K^b expression binding of the H-2K^b tetramer is much stronger, but still Ly49Q-dependent (Fig. 5 A, compare bottom two dot plots). Differential tetramer binding was not due to differences in Ly49Q expression between WT and H-2K^{b}D^b-null mice as pDCs from both mouse strains expressed similar levels of

Ly49Q (Fig. 5 B). These results suggest that in WT pDCs, *cis* interactions between Ly49Q and endogenous H-2K^b are inhibiting binding of soluble H-2K^b tetramer to Ly49Q.

Normal TLR9 expression and pDC activation in Ly49Q-deficient mice

Although the *in vitro* mAb-mediated blocking experiments are consistent with the phenotype of Ly49Q-null pDCs *in vitro* and *in vivo*, the defect in TLR-9-induced cytokine production might be the result of factors other than the absence of Ly49Q. First, Ly49Q deficiency may have caused down-regulation of TLR-9. To exclude this possibility TLR-9 expression was determined by RT-PCR and intracellular flow cytometry given the localization of TLR-9 in endosomes. TLR-9 levels were measured and found to be similar in both Ly49Q-WT and null pDCs (Fig. 6 A, and data not shown), suggesting that Ly49Q-null pDCs have an equal potential to bind CpG-ODN. Second, the almost complete lack of IFN-α secretion by Ly49Q-null pDCs may be due to an inability of the cells to become fully activated. Downstream activation events in pDCs both *in vivo* (Fig. 6 B) and *in vitro* (Fig. 6 C) after CpG-ODN challenge were assessed by flow cytometry analysis of CD86 and MHC class II surface expression, and found to be normal. The percent of pDC expressing CD86 and the level of CD86 on pDCs were similar between WT and Ly49Q-null pDCs after CpG treatment, even over an extended range of doses (Fig. 6 B,C). Similar observations were made for MHC class II (data not shown). Collectively, these data suggest that the signaling pathways downstream of TLR-9 that induce expression of CD86/MHC-II are

different from those that lead to IFN-α production such that Ly49Q signaling modulates only the latter responses in pDCs.

TLR-7-mediated cytokine secretion is defective in Ly49Q-null mice.

To further characterize the extent of the defect in Ly49Q-null pDC cytokine responses, a distinct TLR pathway was evaluated. In addition to high levels of TLR-9, pDC also express TLR-7, which can recognize ssRNA motifs represented by such viruses as influenza and vesicular stomatitis virus. Similar to CpG-ODN stimulation, infection of isolated pDCs with influenza virus resulted in high levels of secreted IFN-α as previously reported (32). Ly49Q-null pDCs produced five-fold less IFN-α and three-fold less IL-12 than WT pDCs in response to influenza (Fig. 7 A, B). Active and inactivated influenza virus treatments gave similar results (data not shown). In contrast, CD86 and class II MHC up-regulation of isolated pDCs in response to a TLR-7 agonist was normal in Ly49Q-null pDCs (Fig. 7 C, and data not shown). Therefore, TLR-7-induced cytokine secretion is also attenuated in Ly49Q-null pDCs.

Ly49Q-deficient mice exhibit diminished protection to MCMV infection

The defect in IFN-α production seen in Ly49Q-null mice after TLR-9 stimulation may have deleterious consequences during immune responses against pathogens that are detected through TLR-9. Previous studies have reported that TLR-9 deficiency promotes increased susceptibility to MCMV infection, as manifested by higher viral loads and defective production of multiple cytokines, including type I IFN (80, 263). As such, the defect in TLR-9-dependent IFN-α production observed in Ly49Q-null mice may also lead to diminished pDC

responses and altered innate immunity to DNA viruses like MCMV. In keeping with this, purified Ly49Q-null pDCs produce approximately three-fold less IFN-α than WT pDCs in response to overnight MCMV infection *in vitro* (Fig. 8 A). The MCMV/pDC co-culture experiment confirms, independently of CpG-ODN, that pDC from Ly49Q-null mice display defective IFN-α production when triggered through TLR-9.

To confirm these findings *in vivo*, Ly49Q-null mice were infected with MCMV. As shown in Figure 8 B, splenic viral titers were consistently higher in Ly49Q-null mice versus WT control animals. While not all experiments showed a statistically significant difference between the two cohorts (e.g., day 3, 6000 PFU; Fig. 8 B), the detection of impaired viral immunity may be complicated by the susceptible Ly49H$^-$ 129 background of these mice (210). Because the *Ly49h* gene conferring resistance to MCMV in B6 mice (210, 264), is less than 300 kb away from the targeted *Ly49q*^{lox} allele these experiments could not be repeated on the MCMV-resistant B6 background at this time. Notwithstanding this, the majority of experiments did reveal a statistically significant increase in Ly49Q-null MCMV titers when performed using either an early time point (1.5 days post-infection), a low initial MCMV inoculum (600 PFU), or both (Fig. 8 B). In agreement with our findings, TLR-9-null mice on a susceptible background (Ly49H$^-$ BALB/c) display a similarly small, but significant increase in splenic viral titers after MCMV infection (263). Serum IFN-α and IL-12 levels peak at 36 hours post MCMV-infection. At this time point Ly49Q-null mice display normal level of IFN-α (Fig. 8 C) and slightly decreased IL-12p70 levels (Fig. 8

D). This suggests that in addition to pDCs other cell types can contribute to serum IFN-α/IL-12 levels by 36 hours post-infection. Collectively, these experiments demonstrate that Ly49Q-null pDCs exhibit a functional deficiency in IFN-α production in response to MCMV infection *in vitro*, and this translates into an enhanced susceptibility of Ly49Q-null mice to MCMV infection *in vivo*.

5. DISCUSSION

Ly49q is a highly-conserved *Ly49* found in all mouse strains characterized to date (237). Ly49Q is also unique in being expressed on pDCs, in contrast to all other Ly49. We have previously shown using the BWZ lacZ-based reporter cell assay that H-2K^b is a high affinity ligand for Ly49Q (257). Here, we confirm the specificity of Ly49Q for H-2K^b in a functional assay (IFN-α secretion) using fresh *ex-vivo* pDCs. Remarkably, Ly49Q: H-2K^b interactions appear to be required for optimal TLR-7- and TLR-9-dependent stimulation of cytokine production by pDCs as evidenced by the drastic reduction in CpG-mediated IFN-α and IL-12 secretion upon mAb-mediated blockade of either Ly49Q receptor or its cognate ligand, H-2K^b. In further support of this hypothesis, purified pDCs cultured in the presence of low concentrations of CpG-ODN produced high levels of IFN-α when stimulated on immobilized Ly49Q mAb or H-2K^b/Ig fusion protein but not in the presence of CpG-ODN alone. Taken together, these findings demonstrate that Ly49Q:MHC I interactions positively regulate TLR signals and subsequent cytokine production in pDCs.

In confirmation of the above *in vitro* experiments, Ly49Q-null mice show a severe defect in systemic IFN-α levels (75-100% reduction) upon challenge with CpG-ODN. This defect is likely due to the hypo-responsiveness of Ly49Q-null pDCs, as purified pDCs from these mice exhibit similarly decreased IFN-α responses upon CpG-mediated stimulation in isolation. Ly49Q-null pDCs also show a defect in the production of IFN-α after overnight culture with MCMV. Both CpG-ODN and MCMV stimulate IFN-α production by pDCs in a TLR-9-

dependent manner (263). However, TLR-9 mRNA and protein expression levels in Ly49Q-null pDCs are normal, as deduced by semi-quantitative RT-PCR and intracellular staining, respectively. The Ly49Q-null defect similarly affects TLR-7, as TLR-7-dependent stimulation of isolated Ly49Q-null pDCs results in highly decreased levels of IFN-α relative to WT pDCs. Notably, in addition to IFN-α levels, Ly49Q-null pDCs also exhibit decreased IL-12p70 levels in response to CpG-ODN or influenza stimulation. Thus, Ly49Q-null pDCs possess an intrinsic cytokine production defect in response to TLR-7 and TLR-9 agonists. Interestingly, however, the activation status of Ly49Q-null pDCs seems normal over a wide range of CpG-ODN concentrations, at least with respect to up-regulation of CD86 and class II MHC surface expression, TNF-α and IL-6 production, morphologic change, and migration within the spleen. This suggests that TLR9 stimulation results in multiple signaling pathways leading to pDC activation, but that Ly49Q regulates only the signal transduction pathway leading to IFN-α secretion.

The phenotype of pDCs from Ly49Q-null mice is similar to that observed for Ly49Q-negative pDCs from WT bone marrow. Omatsu *et al.* found that Ly49Q-negative pDC produced significantly less IFN-α/β than their Ly49Q-positive counterparts when cultured in the presence of CpG-ODN or Sendai virus (111). In contrast, Kamogawa-Schifter *et al.* observed comparable IFN-α production levels from the two pDC subsets *in vitro* after stimulation with CpG-ODN (11). It is not immediately clear why there is a discrepancy in IFN-α production among Ly49Q-positive vs. Ly49Q-negative pDCs in these two studies,

as similar isolation procedures and the same mouse strain (BALB/c) were employed. The data presented in this manuscript agree with the former study, in that the ability of pDCs to produce large amounts of IFN-α appears to depend on Ly49Q expression and function.

The positive regulation of TLR-9 signaling by Ly49Q is at first counterintuitive, given that the ITIM-bearing Ly49Q receptor has the characteristics of an inhibitory Ly49 family member. Similar Ly49 receptors expressed by NK cells undergo tyrosine phosphorylation of their cytoplasmic ITIM in response to receptor engagement, resulting in the recruitment of the SHP-1/2 phosphatases (265). Ly49-associated SHP-1/2 are then thought to antagonize stimulatory signals initiated through ITAM-bearing adaptor molecules such as DAP-12. For example, the ITIM-bearing Ly49G receptor has been demonstrated to suppress DAP-12-dependent Ly49D-mediated cytotoxicity and cytokine production (266). Notably, the ability of the Ly49Q ITIM to associate with SHP-1/2 is intact, at least in reconstitution experiments using transfected cell lines (35). Thus, if Ly49Q is an inhibitory receptor, how does it function to relay TLR-7/-9-induced signals or amplify TLR-7/-9-mediated signal transduction? Interestingly, there are clues from experiments performed using DAP-12-null mice that may offer a possible mechanism. Specifically, DAP-12-null pDCs were reported to produce significantly more IFN-α following CpG-ODN stimulation (267), as opposed to the decreased response of Ly49Q-null mice. The resulting conclusion was that DAP-12 may negatively regulate TLR-7/-9 signal transduction and, in turn, that DAP-12-coupled receptors on pDCs may inhibit IFN-α secretion upon

cross-linking. In support of this hypothesis, the pDC-expressed and DAP-12-associated surface proteins Nkp44 and Siglec-H do indeed inhibit CpG-induced IFN-α secretion upon mAb-mediated ligation (33, 49). In this respect, macrophages and pDCs are functionally similar, as macrophages from DAP-12-null mice produce more cytokines following TLR engagement (32). Furthermore, the DAP-12-coupled TREM2 surface receptor appears to deliver constitutive inhibitory signals in macrophages (268).

The apparent stimulatory role of Ly49Q in pDCs could be due to antagonism of inhibitory DAP12 signal transduction by SHP-1/2 recruited as a result of Ly49Q/MHC interaction. In this model, DAP-12 signaling would be constitutive like TREM2 function in macrophages (33). Although the convergence point between the DAP-12 and TLR-9 signaling pathways is unclear, another possibility is that downstream Ly49Q signaling molecules may directly regulate the TLR-9 signaling cascade independently of DAP-12 function. A third mechanism behind Ly49Q function may include an alternative signaling pathway found in pDCs, one which is distinct from canonical inhibitory Ly49 signaling in NK cells and does not involve the recruitment of tyrosine phosphatases. Evidence for this possibility includes Ly49Q-transfected macrophage cell lines, which show an increase in tyrosine-phosphorylated proteins in whole cell lysates after mAb-mediated cross-linking of Ly49Q (35).

The mechanistic link between Ly49Q function and IFN-α secretion is unknown. However, an interesting parallel may exist between NK cells and pDCs in this regard. Two reports have suggested that mature splenic NK cells lacking

all self-specific MHC-binding inhibitory receptors (NKG2A and Ly49C/I in B6 mice) are hypo-responsive with respect to the killing of target cells and the production of IFN-γ (255, 256). Thus, B6-strain NK cells expressing NKG2A and/or Ly49C/I have been referred to as being 'licensed' or 'armed'. It is tempting to speculate that pDC like NK cells may also require the expression of at least one self-specific MHC-binding inhibitory receptor to become fully functional IFN-α producers. This hypothesis is consistent with the hyporesponsiveness observed for immature Ly49Q-negative pDC (111). Since Ly49Q binds with high affinity to H-2K^b, a self-specific determinant expressed in 129-strain Ly49Q-null mice, the lack of a self-MHC specific inhibitory signal during pDC development in Ly49Q-null mice may render these cells anergic. However, the recapitulated phenotype using WT pDC in mAb blocking experiments (Fig. 1) argue against Ly49Q-null pDCs being anergic.

Type I IFN is indispensable for resistance to a wide range of viruses, as shown by studies using IFN-α/βR-null mice (269). Ly49Q-null mice, which are on the Ly49H$^-$ 129 background, also display a severe IFN-production defect as well as a consistent and statistically significant increase in susceptibility to MCMV. However, the susceptibility of Ly49Q-null mice to virus infection is not as profound as that observed in IFN-α/βR-null mice. There are several possible reasons for this observation. First, although the magnitude of the IFN-α response to CpG-ODN challenge in WT mice far exceeds that of Ly49Q-null mice, the latter still yield up to 15-25% of WT serum IFN-α levels. This lower yet detectable IFN-α production may be sufficient to mount resistance to viral

infection. This hypothesis is consistent with the phenotype of Ly49H$^+$ B6 mice, which produce low levels of IFN-α (32), but are nonetheless sufficient for protection against MCMV and other viruses. In agreement with our study, TLR-9-null mice on a BALB/c (MCMV susceptible; Ly49H$^-$) background also only show a small increase in susceptibility (263), as would be expected if Ly49Q does indeed regulate TLR signaling. Furthermore, normal systemic IFN-α levels were observed in Ly49Q-null mice 36 h after MCMV infection, despite their defective IFN-α responses to synthetic CpG-ODN. Therefore, while pDC cytokine production is compromised in Ly49Q-deficient mice, and does confer limited susceptibility to viral infection, the residual capacity of these mice to produce anti-viral cytokines probably affords sufficient protection. Whether these residual cytokines are made by Ly49Q-null pDCs or other cell types is not clear.

Based on the data generated from mAb-mediated blocking assays and functional analyses of Ly49Q-null pDC *in vitro*, as well as *in vivo* analyses of Ly49Q-null mice, we conclude that pDC function, in particular IFN-α secretion, is regulated by class I MHC. The ability of pDCs to recognize MHC I molecules is imparted by the lectin-like Ly49Q receptor, which in turn potentiates the capacity of pDCs to produce cytokines in response to TLR-7 and TLR-9 agonists. Loss of this self-recognition mechanism results in severe defects in IFN-α and IL-12 production by pDCs. Thus, Ly49Q-MHC interactions positively regulate pDC cytokine production triggered through TLR. Therefore, the down-regulation of class I MHC by certain viruses like MCMV could also result in the attenuation of pDC anti-viral defense mechanisms in addition to evasion of CD8$^+$ T cell

responses. Interestingly, despite bearing a consensus ITIM sequence, the function of Ly49Q on pDCs appears to be stimulatory in nature. While this is somewhat paradoxical, it is nonetheless consistent with the reported counter-regulatory function of DAP-12-coupled receptors in pDCs, which appear to be inhibitory.

6. ACKNOWLEDGEMENTS

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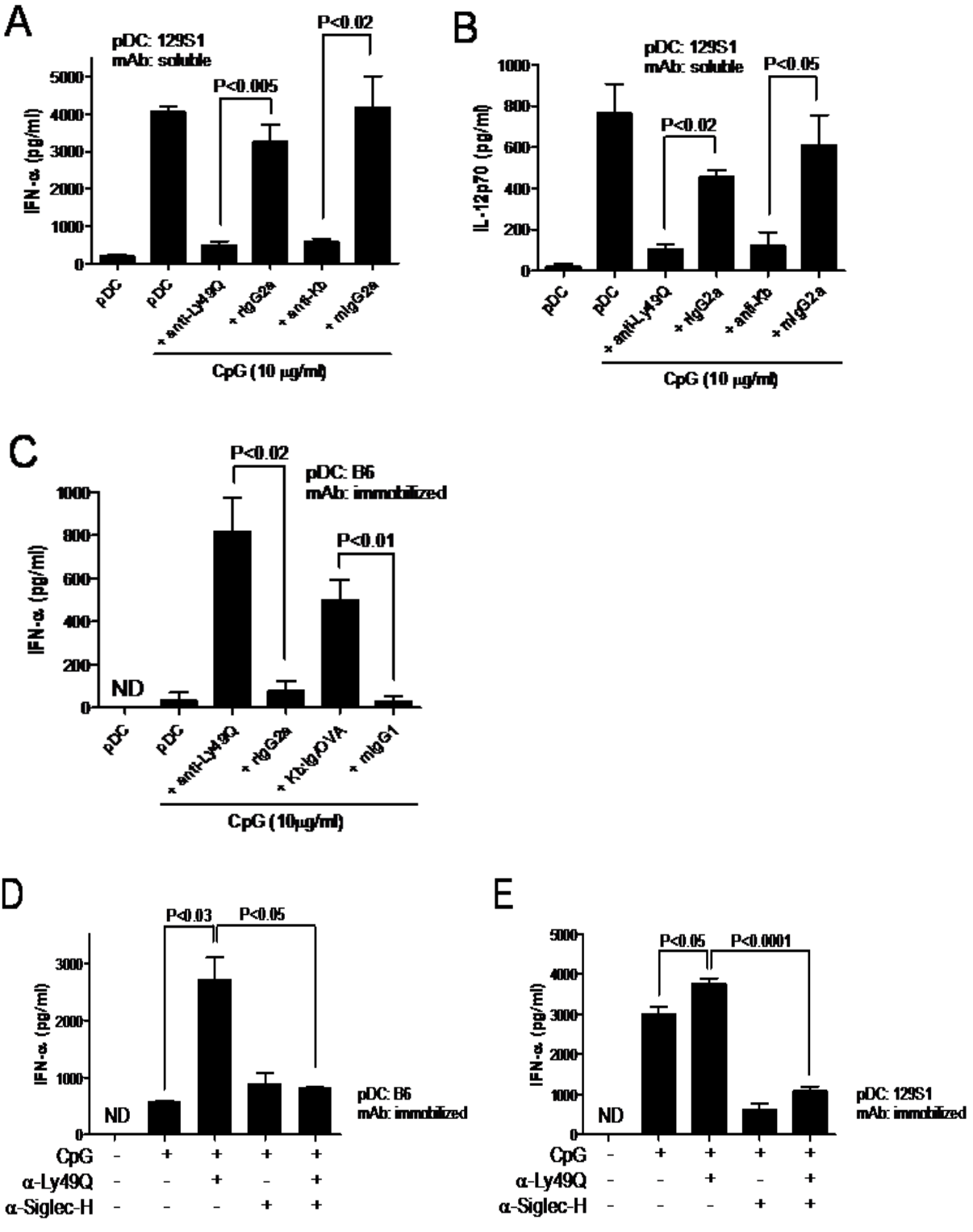
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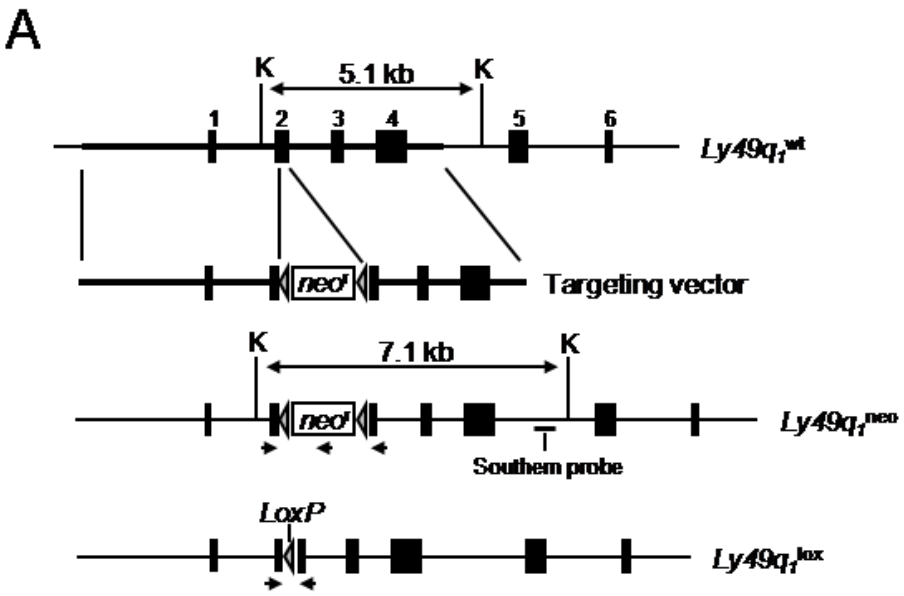
Figure 1: Ly49Q-H-2K^b interactions are necessary for pDC cytokine secretion after TLR-9 stimulation. (A) Splenic pDCs from 129S1 mice were isolated using mPDCA-1 microbeads and cultured overnight in the presence or absence of CpG-ODN and the indicated mAb. The supernatant was assayed by ELISA for IFN-α or (B) IL-12p70. (C-E) Splenic pDCs from B6 (C and D) or 129S1 mice (E) were isolated with mPDCA-1 microbeads and cultured overnight in wells that had been precoated with the indicated mAb or recombinant MHC molecule in the presence or absence of 10 μg/ml CpG. The supernatant was assayed by ELISA for IFN-α. Isotype control Ab for anti-Ly49Q, anti-H-2K^b, and K^b:Ig/OVA are rIgG$_{2a}$, mIgG$_{2a}$, and mIgG$_1$, respectively. Anti-Ly49Q mAb 2E6 was used for these experiments. Data are presented as the mean of triplicate samples (error bars, s.d.). Results are representative of at least three independent experiments. ND, not detectable.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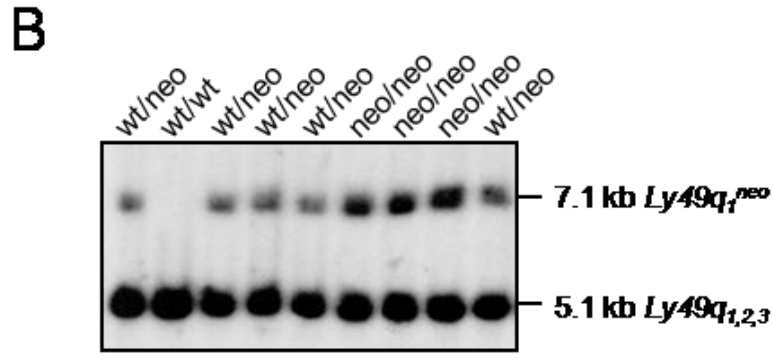
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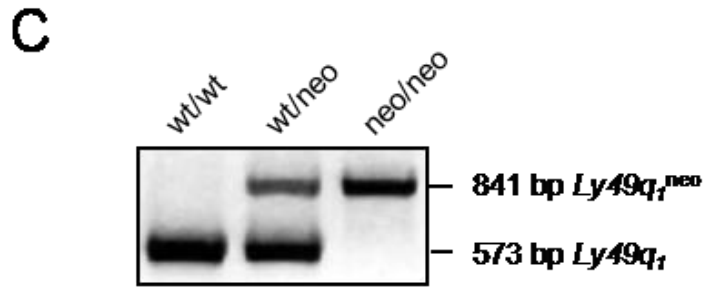
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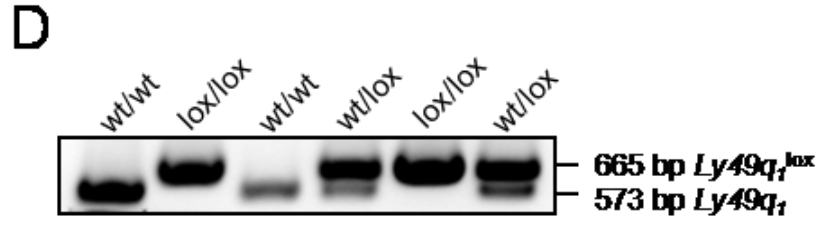
wt/wt
wt/neo
neo/neo
841 bp Ly49q1neo
573 bp Ly49q1


D
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lox/lox
wt/wt
wt/lox
lox/lox
wt/lox
665 bp Ly49q1lox
573 bp Ly49q1

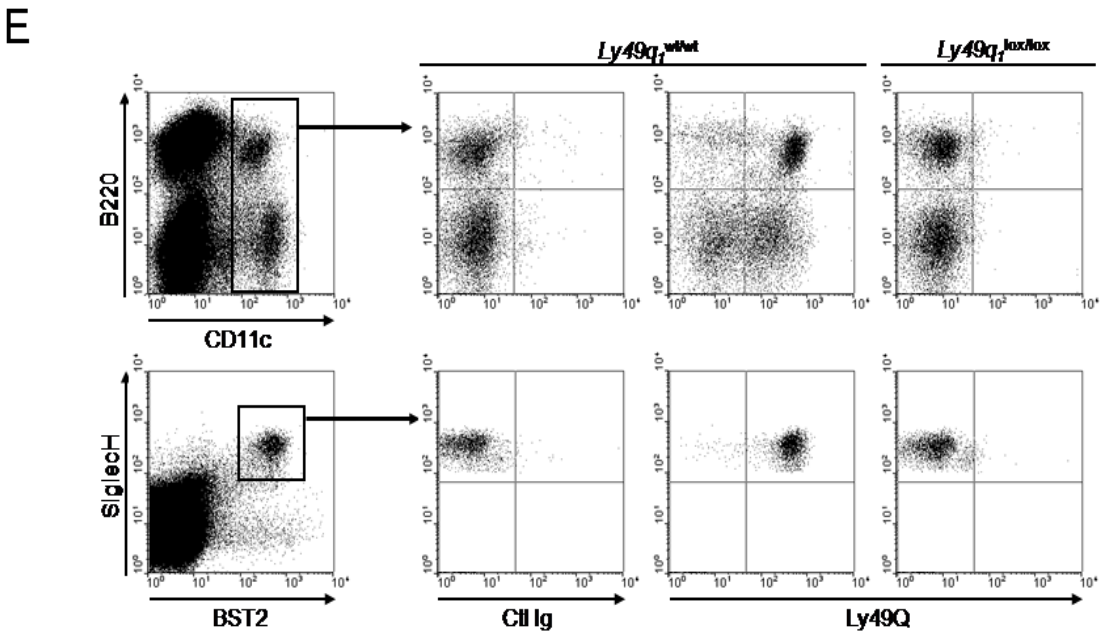
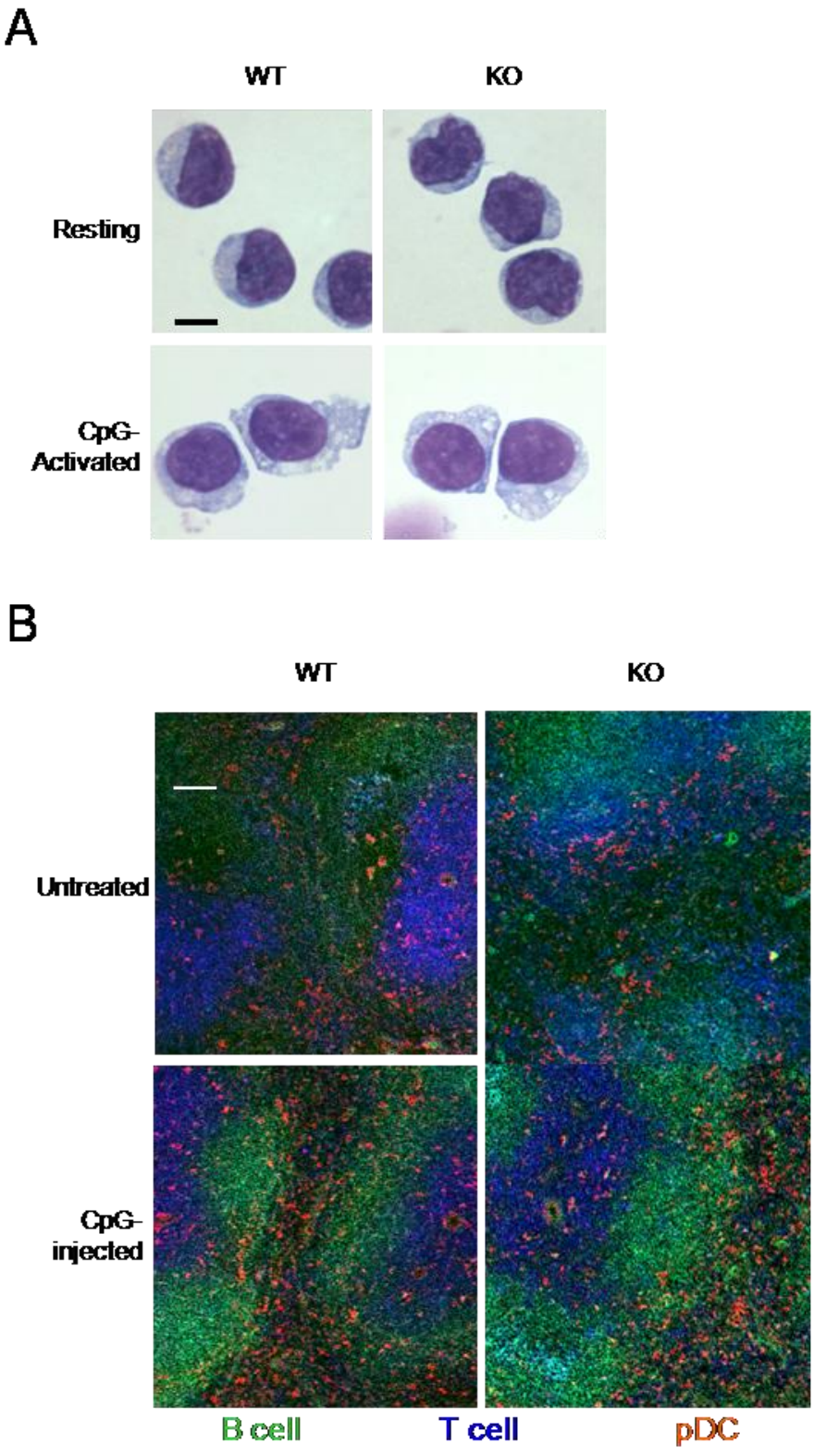


Figure 2. Generation of Ly49Q-null mice. (A) *Ly49q*$_1$ gene disruption strategy. A 10 kb segment encompassing exons 1-4 was cloned by recombineering from BAC DNA, and a floxed *PGK-Neo*^{r} (*neo*^{r}) cassette was then inserted into exon 2. After electroporation and selection, an ES clone possessing the predicted KpnI (K) fragment as verified by Southern blotting was electroporated with CMV-Cre plasmid and the deletion of *PGK-Neo*^{r} was confirmed by PCR. Both *Ly49q*$_1^{neo}$ and *Ly49q*$_1^{lox}$ mice were created. PCR primers are shown by arrowheads. Boxes denote exons and the location of the Southern probe is underlined. (B) Southern blot analysis. Genomic tail DNA of pups from *Ly49q*$_1^{neo/wt}$ parent mice was digested with KpnI and analyzed by Southern blot with the probe depicted in (A). Note that the probe detects identical fragments from *Ly49q*$_1$, *q*$_2$, and *q*$_3$ genes. (C) PCR analysis of tail DNA from mice in (B). To eliminate confusion caused by *Ly49q*$_2$ and *q*$_3$ genes, *Ly49q*$_1$-specific primers were designed. These primers were used to analyze tail DNA by PCR and to differentiate between *Ly49q*$_1^{wt}$ and *Ly49q*$_1^{neo}$ alleles. (D) PCR analysis of pups from *Ly49q*$_1^{lox/wt}$ parent mice. Tail DNA was PCR-amplified with primers flanking exon 2 to differentiate *Ly49q*$_1^{wt}$ and *Ly49q*$_1^{lox}$ alleles. (E) Lack of Ly49Q protein on dendritic cells derived from Ly49Q-null mice. Ly49Q-null and WT splenocytes were stained with a combination of mAb to CD11c/B220 (upper panel) or Siglec-H/BST-2 (lower panel), and Ly49Q-specific mAb NS-34 or an isotype control. Ly49Q mAb staining intensity relative to isotype control mAb on gated cells is shown.


A
WT
KO
Resting
CpG-
Activated
B
WT
KO
Untreated
CpG-
injected
B cell
T cell
pDC

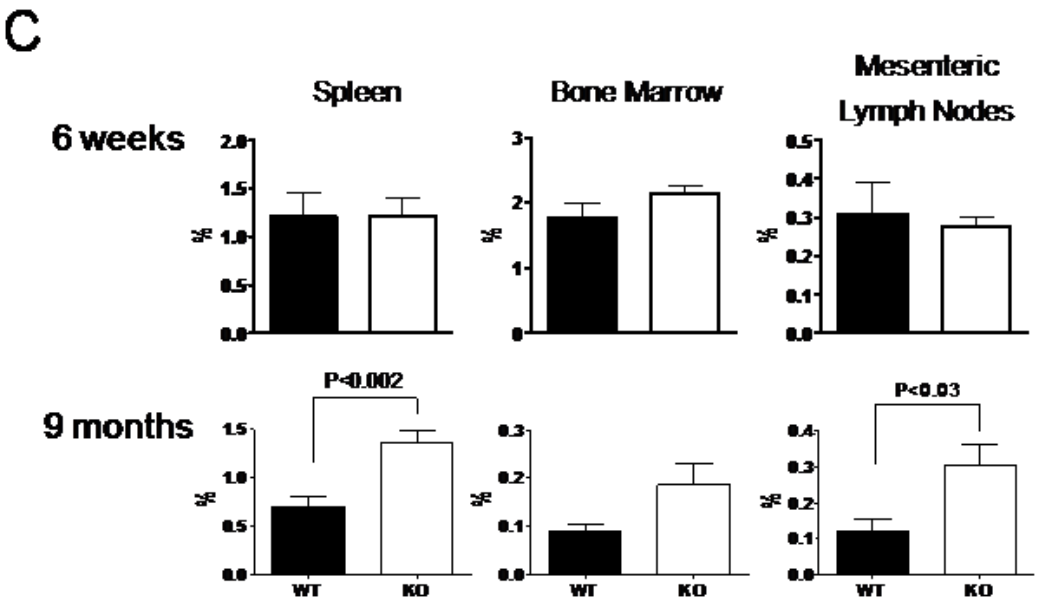
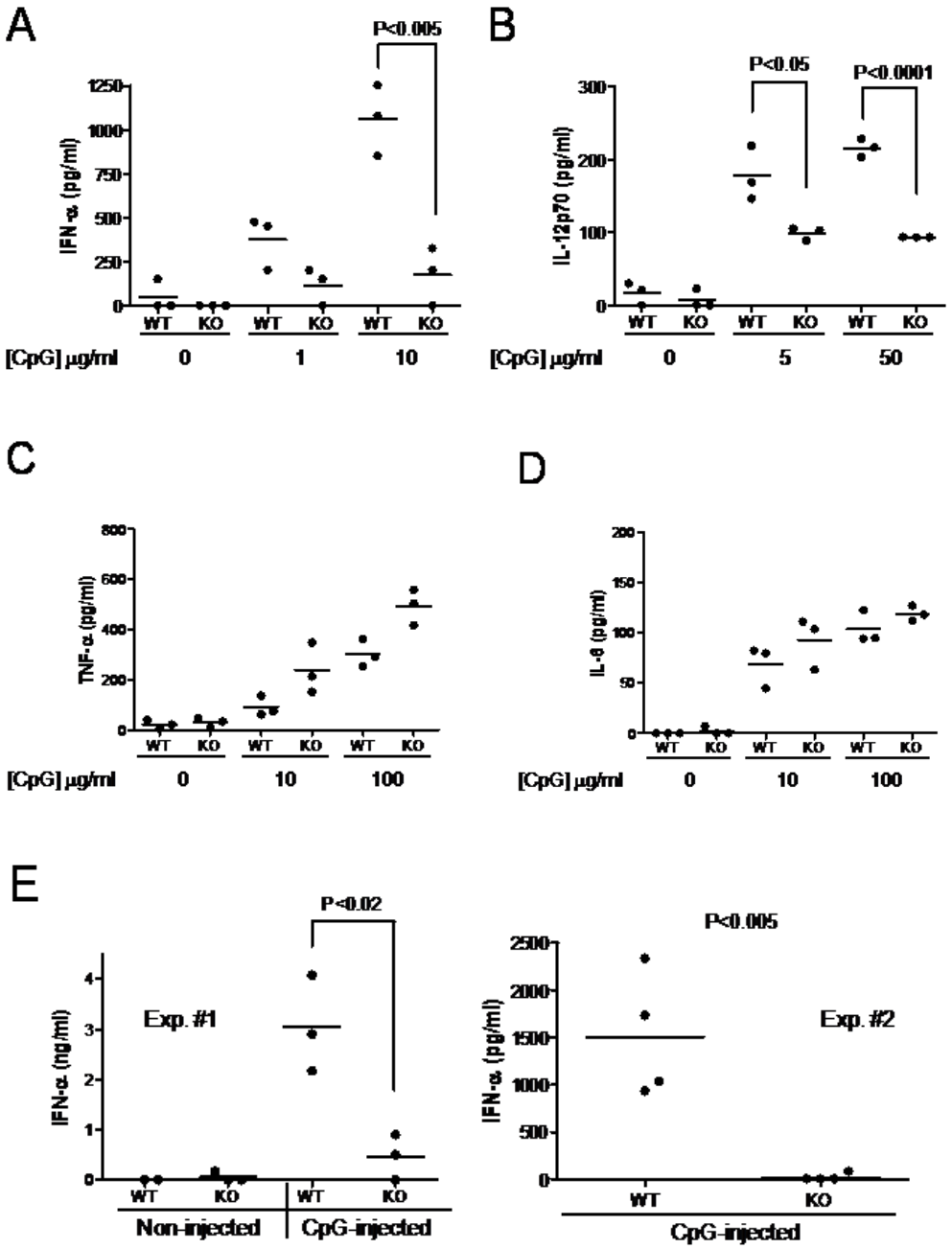


Figure 3. Age-dependent increase in pDCs proportion in Ly49Q-null mice**.** (A) pDC size and morphology. pDCs were sorted as $CD11c^{+}B220^{+}CD11b^{-}DX5^{-}CD3^{-}CD19^{-}$ cells from the spleen and cytocentrifuged on microspcope slides directly as resting pDCs or after incubation with 3 μg/ml of CpG for 16h. Slides were then stained with Giemsa solution. The scale bar represents 5 μm. (B) Localization of splenic pDCs. Sections of spleen isolated from untreated young mice or mice i.v. injected with CpG (10 μg/ml) 24 h earlier were stained with fluorescently labeled mAb to CD19 (B cells, green), CD3 (T cells, blue) and BST-2 (pDCs, red). The scale bar represents 50 μm. (C) Frequency of pDCs in primary and secondary lymphoid organs. Shown are the proportion of pDCs defined as $CD11c^{+}Siglec\text{-}H^{+}$ cells in lymphoid organs of *Ly49q*$^{+/+}$ (■) and *Ly49q*$^{lox/lox}$ (□) mice (6 weeks old, n=3; 9 months old, n=6). A similar pattern was observed with BST-2 staining (data not shown). Data are presented as the mean of individual mice (error bars, s.d.). Results are representative of at least three independent experiments, except for (C) (9 months), which was done twice.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IFN-α (pg/ml)
1250
1000
750
500
250
0
WT KO WT KO WT KO
[CpG] µg/ml 0 1 10
B
P<0.05
P<0.0001
IL-12p70 (pg/ml)
300
200
100
0
WT KO WT KO WT KO
[CpG] µg/ml 0 5 50
C
TNF-α (pg/ml)
800
600
400
200
0
WT KO WT KO WT KO
[CpG] µg/ml 0 10 100
D
IL-6 (pg/ml)
200
150
100
50
0
WT KO WT KO WT KO
[CpG] µg/ml 0 10 100
E
P<0.02
IFN-α (ng/ml)
4
3
2
1
0
Exp. #1
WT KO WT KO
Non-injected CpG-injected
P<0.005
IFN-α (pg/ml)
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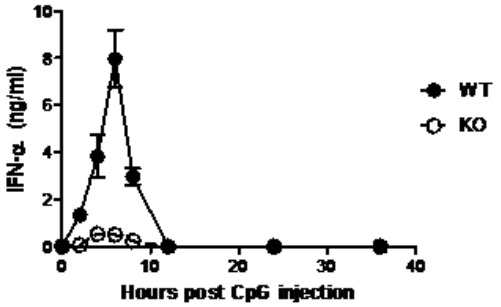
2000
1500
1000
500
0
Exp. #2
WT KO
CpG-injected

F



G

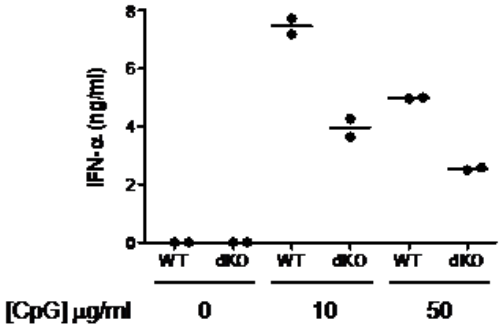


Figure 4. Ly49Q-null pDCs display an IFN-α production defect in response to CpG-ODN. (A) IFN-α production by isolated pDCs after CpG-ODN stimulation. pDCs were isolated with mPDCA-1 microbeads from Ly49Q-null and WT mice and cultured overnight in the presence of the indicated concentrations of CpG-ODN. Culture supernatants were assayed by ELISA for IFN-α, (B) IL-12p70, (C) TNF-α, or (D) IL-6. (E) Induction of serum cytokines by CpG-ODN injection. Ly49Q-null and WT littermates were injected with CpG-ODN + DOTAP and blood samples were taken after 6h for IFN-α ELISA. Uninjected mice served as controls. Each dot represents a single mouse. (F) Serum IFN-α time-course after CpG-ODN stimulation. Ly49Q-null and WT littermates were injected with CpG-ODN + DOTAP and then blood was collected periodically over 36h. Serum IFN-α levels were deduced by ELISA from three mice of each genotype for each time-point. Data are representative of experiments done at least three times independently. (G) pDCs were isolated from H-2K^{b}D^b-null or WT (B6) mice and treated as in (A).

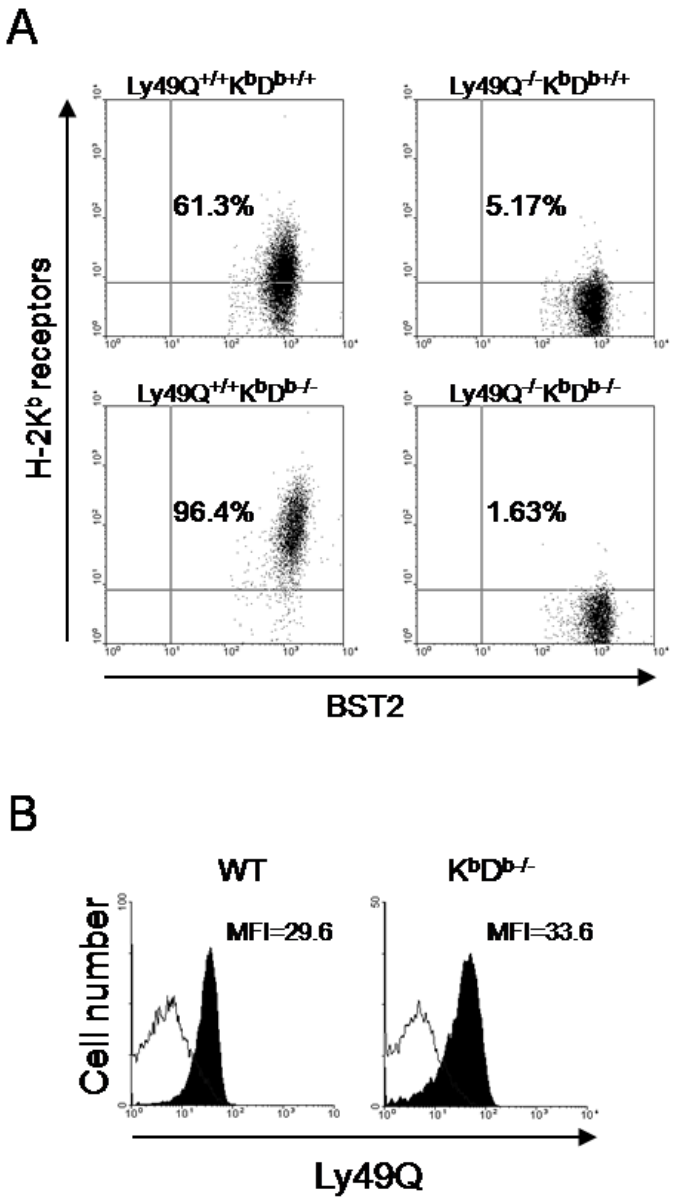


Figure 5. Ly49Q binds to H-2K^b in *cis*. (A) pDCs were isolated from the spleens of mice with the indicated genotypes with mPDCA-1 microbeads. Isolated pDCs were stained with PE-H-2K^b/OVA tetramer and APC-anti-BST2 mAb and then analyzed by flow cytometry. Cells are gated on pDCs. The percentage of cells positively staining with PE-H-2K^b/OVA is shown. (B) Ly49Q levels on MHC-deficient pDCs. Splenocytes from the indicated strains (WT is B6) were stained for BST-2, SiglecH, and Ly49Q (NS34). The level of Ly49Q expression on BST-2$^+$SiglecH$^+$ cells is shown (filled histogram). The open histogram represents the secondary reagent alone.

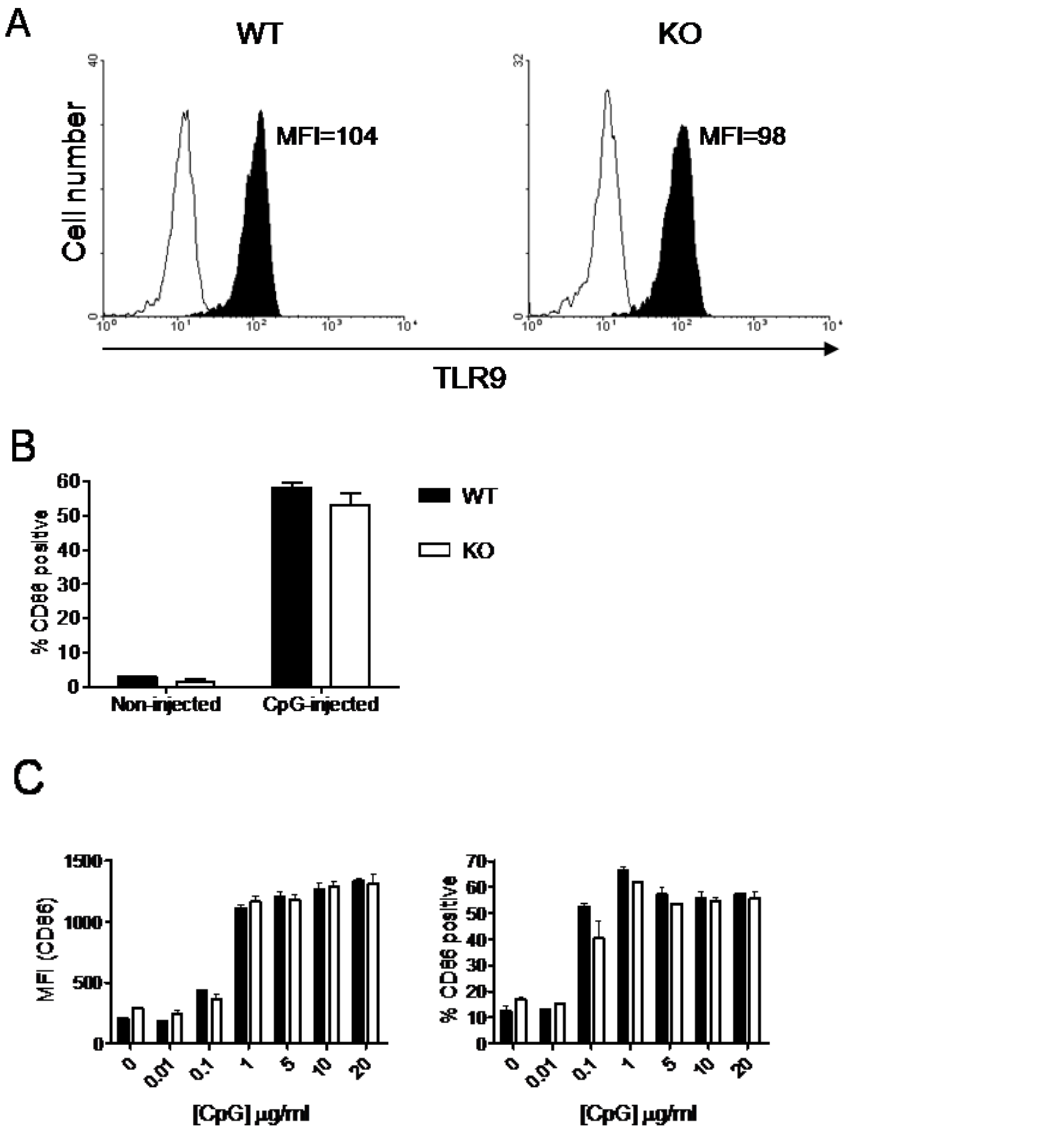


Figure 6. Normal TLR-9 and CD86 expression by Ly49Q-null pDCs. (A) Splenocytes from Ly49Q-null and WT mice were stained for Siglec-H and BST2, then permeabilized and stained for intracellular TLR-9 or with an isotype control mAb. TLR9 (black histogram) or control (white histogram) expression on Siglec-H^+BST2^+ cells is shown. (B) *In vivo* activation of pDCs by CpG-ODN. Ly49Q-null and WT littermates were injected with CpG-ODN + DOTAP. Uninjected mice served as controls. After 6h the splenocytes were isolated, stained for Siglec-H, BST-2, and CD86, and analyzed by flow cytometry. The percent of $CD86^+$ pDCs after 6h is shown. (C) *In vitro* activation of pDCs by CpG-ODN. pDC from Ly49Q-null and WT mice were isolated with mPDCA-1 microbeads and cultured overnight in the presence of the indicated concentrations of CpG-ODN. The following day the $CD11c^+B220^+$ cells were analyzed by flow cytometry for CD86 surface expression. Mean fluorescence intensity (MFI) and percent positive cells are shown in the left and right graphs, respectively, as the mean of triplicate cultures ± s.d. Data are representative of at least three independent experiments.

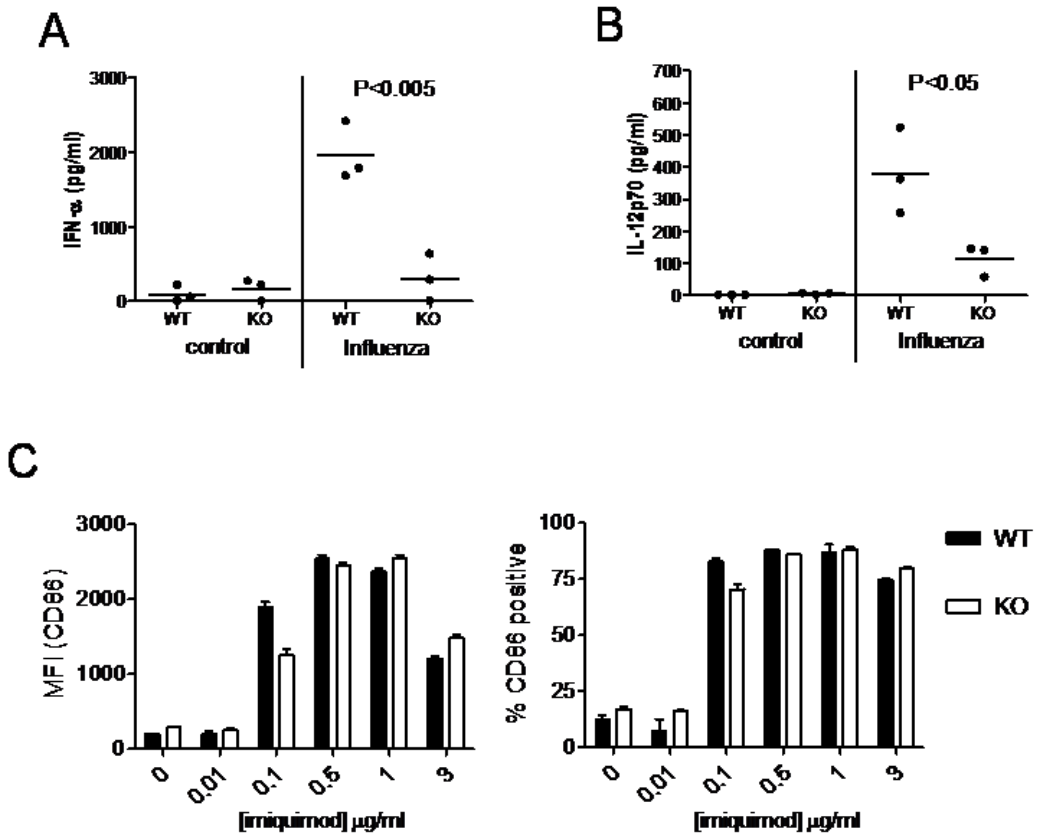
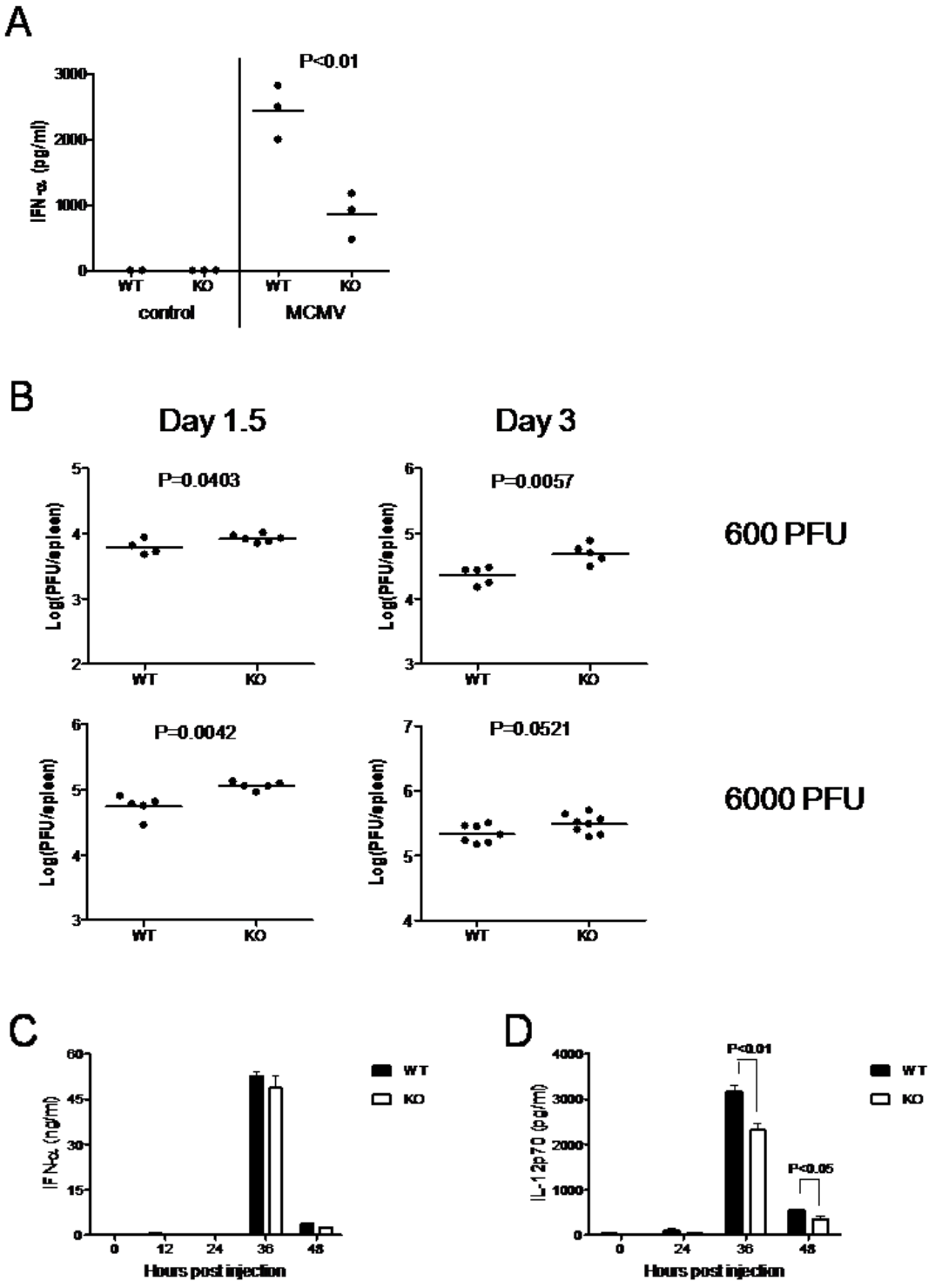


Figure 7. TLR-7 responses are defective in Ly49Q-null mice. (A) *In vitro* activation of pDCs by influenza virus. pDCs from Ly49Q-null and WT mice were isolated using mPDCA-1 microbeads and cultured overnight in the presence or absence of 20 HA units of influenza virus. Culture supernatants were assayed by ELISA for IFN-α or (B) IL-12p70. Each dot represents a single mouse. (C) *In vitro* activation of pDC by imiquimod. pDCs from Ly49Q-null and WT mice were isolated using mPDCA-1 microbeads and cultured overnight with the indicated concentrations of imiquimod. The following day $CD11c^{+}B220^{+}$ were stained with FITC-anti-CD86 and analyzed by flow cytometry for CD86 surface expression. Mean fluorescence intensity (MFI) and percent positive cells are shown in the left and right graphs, respectively, as the mean of triplicate cultures ± s.d. Data are representative of at least three independent experiments.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2000
1000
0
WT
KO
WT
KO
control
MCMV
B
Day 1.5
Day 3
P=0.0403
P=0.0057
Log(PFU/spleen)
600 PFU
P=0.0042
P=0.0521
6000 PFU
C
IFN-α (ng/ml)
60
45
30
15
0
0
12
24
36
48
Hours post injection
WT
KO
D
IL-12p70 (pg/ml)
4000
3000
2000
1000
0
P<0.01
P<0.05
0
24
36
48
Hours post injection
WT
KO

Figure 8. Reduced anti-MCMV responses by Ly49Q-null mice**.** (A) Decreased MCMV-induced IFN-α secretion by pDCs *in vitro*. Splenic pDCs were isolated with mPDCA-1 microbeads and cultured overnight in the presence of 200 PFU/ml of MCMV. Supernatants were harvested the following day. Supernatants were assayed for IFN-α levels by ELISA. (B) Greater MCMV proliferation in Ly49Q-null mice. Ly49Q-null and WT mice were challenged with 600 or 6000 PFU MCMV. After 1.5 or 3 days spleens were harvested and viral titer was determined using BALB/c MEF. Each dot represents a single mouse. Data are representative of at least three independent experiments. (C and D) Littermates of the indicated genotypes were infected with 6000 PFU MCMV i.p. and serum samples were taken at the indicated timepoints post-infection. IFN-α (C) or IL-12p70 (D) levels were determined by ELISA (n = 4 per timepoint). The means of individual mice ± s.d. are shown.

CHAPTER 4: DISCUSSION AND OPPORTUNITIES FOR RESEARCH

We have identified the MHC class I molecule H-2K^b as an endogenous Ly49Q ligand (257). This is both an expected and interesting finding. Due to homology with other inhibitory Ly49 receptors, the identity of the ligand for Ly49Q was expected to be an MHC class I molecule. This was formally confirmed with reporter cell recognition of recombinant H-2K^b:peptide loaded complex and H-2K^b tetramer staining of Ly49Q on *ex vivo* pDCs (257). The MHC class I molecule, H-2K^b is expressed on essentially all hematopoietic cells of C57BL/6 mice. However, we were not able to detect reporter cell stimulation on *ex vivo* T and B cell suspensions from primary and secondary lymphoid organs from C57BL/6, which express H-2K^b (257). We initially explained our "no stimulation on lymphoid cell types" findings by suggesting the possibility of variation in the level of H-2K^b expression levels as well as the presence of co-stimulatory and adhesion molecules on stimulating vs. non-stimulating cell types (257). Recent findings by Kamogawa-Schifter's group support our rationale on the nature of Ly49Q recognition of H-2K^b. They utilized a Ly49Q-FcM fusion protein to stain for potential ligands on stimulator cells. Similar to our findings, they were unable to detect any ligands on *ex vivo* splenocytes. However, upon splenocyte activation with CpG, they observed Ly49Q fusion binding to activated CD19$^+$ B cells (258). Interestingly, they observed clustering of H-2K^b on CpG activated B cell surfaces and co-immunoprecipitation of SHP-1 with Flag-tagged Ly49Q when Ly49Q-transfected Chinese hamster ovarian (CHO) cells were incubated with CpG stimulated B cells (258). This may explain the inability of

resting LN cells to stimulate BWZ.Ly49Q reporter cells as clustering of MHC I was absent.

We used unfractionated $CD11c^{+}$ in our reporter assay, which include both mDCs and pDCs. Since Ly49Q receptor resides on pDCs, it might be of interest to test stimulation of BWZ.Ly49Q cells with pDCs in the reporter assay. However, we would not expect stronger stimulation of BWZ.Ly49Q by pDCs in comparison to mDCs. This is due to the variation in surface expression of costimulatory molecules of both DC subsets in the resting phase. As outlined in the literature review (Chapter 1), resting mDCs are better APCs due to constitutive expression levels of costimulatory/adhesion molecules, assuming these accessory molecules play a role in the immunology synapse of ligand bearing cell : reporter cell. On the other hand, resting pDCs contain low levels of such molecules and thus may not stimulate BWZ.Ly49Q cells, much like resting LN cells.

Related to the above issues describing unexpected ligand recognition, one set of our transfectants – BWZ.Ly49Q^{B6} reporter cells were more weakly stimulated by DCs than BWZ.Ly49Q^{BALB} cells. Both sets of BWZ transductants expressed comparable surface levels of chimeric Ly49Q receptor, thus ruling out reporter cell differences. However, low levels of unknown, endogenous Ly49Q ligand could be expressed on BWZ cells. These may represent weak Ly49Q:H-2^{k} interactions that set the threshold of receptor-ligand engagement. Both BWZ.Ly49Q^{BALB} and BWZ.Ly49Q^{B6} may recognize MHC I molecules of H-2^{k} haplotype *in cis* and therefore bind H-$2K^{b}$ with varying affinities (more weakly in the case of BWZ.Ly49Q^{B6}). Binding of Ly49 with an MHC I from a different

haplotype (unnatural pariring) is through to have risen from the derivation of man made inbred mice strains from diverse geographical locations resulting in alerations of receptor-ligand interactions. This may explain Ly49Q expression on BALB/c pDC since it does not express the H-2K^b haplotype. Whether BALB/c Ly49Q functions analogously to B6 Ly49Q remains open to investigation.

H-2K^b recognition by Ly49Q appears to be peptide dependent, as MHC Ia lacking peptide did not stimulate BWZ.Ly49Q. We have only used the ovalbumin SINFEKL peptide to load H-2K^b:Ig molecules and observed stimulation of BWZ.Ly49Q and have not tested other peptides. A survey of Ly49 literature demonstrates that Ly49 recognition of MHCIa is peptide dependent, but not peptide specific, as Ly49 binding of MHCIa does not make any direct contact with the peptide in the MHCIa groove (220, 221, 226).

As mentioned in the discussion of our Ly49Q ligand paper, we have not ruled out the possibility of other Ly49Q ligands. Binding assays using Ly49 transfected cells such as our BWZ reporter cell system may be inadequate to detect low-affinity interactions under the threshold of reporter cell activation that are nonetheless biologically relevant. Other alleles of H-2K molecules and other H-2 haplotype molecules may serve as endogenous ligands for Ly49Q, albeit at lower affinities. However, this may not be detectable by our BWZ reporter assay because the BWZ cells are of T cell thymoma origin and endogenously express H-2K. Therefore, this sets a threshold of reporter cell activation whereby sensing and binding of H-2K on stimulator cells by the chimeric receptor on BWZ cells must be higher than endogenously expressed H-2K.

The identification of other endogenous Ly49Q ligands will lend further insight into the nature of Ly49Q:ligand binding and pDC function. Therefore, it is worthwhile to test tetrameric H-2 complexes of other H-2K allelelic variants as well as other H-2 haplotypes for direct binding with *ex vivo* pDCs. For these experiments, pDCs from β_2-m-deficient or other MHC-Ia-deficient mice should be used due to potential for *cis* interactions between Ly49Q and MHC-Ia molecules on pDCs that reduce Ly49Q accessibility to tetramer bearing MHC-Ia. The increased binding of H-2K^b tetramer on H-2D^b/H-2K^b-double deficient pDCs (257) and numerous findings on *cis* binding in Ly49 literature necessitates this measure (270). Notably, differential tetramer binding was not due to differences in Ly49Q expression between wildtype and H-2D^b/H-2K^b-double deficient mice as pDCs from both mouse strains expressed similar levels of Ly49Q (Chapter 3, Figure 5 B). These results suggest that in wildtype pDCs, *cis* interactions between Ly49Q and endogenous H-2K^b are inhibiting binding of soluble H-2K^b tetramer to Ly49Q. Despite these results, it would be difficult to totally exclude *trans* interactions as these interactions *in vivo* could induce Ly49Q internalization. Pharmacological inhibitors of endocytosis and cytoskeletal rearrangement prior to internalization can be included as controls in pDC co-cultures prior to tetramer investigation.

Functionally, our investigations into Ly49Q:H-2K^b interaction revealed that Ly49Q positively regulates TLR-9 induction of IFN-α production (271). We show that Ly49Q: H-2K^b in synergy with TLR-9:CpG activates pDCs. Crosslinking pDCs with platebound anti-Ly49Q or recombinant H-2K^b:Ig/OVA

in the absence of CpG did not elicit any IFN-α production (unpublished observatioins).

To demonstrate that Ly49Q was successfully deleted in our Ly49Q-deficient mice, we analyzed both mDC and pDC populations in Ly49Q-deficient mice and verified the lack of Ly49Q expression by flow cytometry. Interestingly, approximately 50% of $CD11c^{+}/B220^{-}$ cells in our Ly49Q-sufficient mice expressed Ly49Q. This pattern of cytokine expression correlates with mDCs. While Ly49Q is predominatly expressed by pDCs, Toyama-Sorimachi et al. has also reported that resting mDCs from NZB, NZW and 129S1/SvJ mice express moderate levels of Ly49Q (238), which agrees with our observations.

While we have not studied the functional consequences of Ly49Q heterzygosity in our mice, recent work by Toyama-Sorimachi's group show that Ly49Q co-localization with CpG containing endo/lysosomes was detected in both pDCs and peritoneal exudates macrophages from $Ly49Q^{+/-}$ mice. In particular, pDCs and macrophages cells lacking Ly49Q, co-localization did not occur and CpG trafficking structures were impaired (88). This supports our findings of defective cytokine secretion in Ly49Q-deficient mice and also suggests that Ly49Q heterozygous mice are not haploinsufficient.

As outlined in Chapter 3, Figure 3C, there is a greater pDC population in primary and secondary lymphoid organs of 9 months old Ly49Q-deficient mice as compared to wildtype littermates. As further functional experiments were not conducted in these older mice beyond assessing pDC hematopoiesis, we cannot speculate as to the reason why these mice harbor a higher pDC population. One

possibility could be an increased recruitment of pDCs to the lymphoid organs by unknown mechanisms to compensate for the IFN-α defect due to the absence of Ly49Q.

In addition to IFN-α, we assessed the ability of wildtype and Ly49Q deficient pDCs to produce other known pDC cytokines. Parallel to IFN-α, we observed defective IL-12 production by Ly49Q-deficient pDCs in comparison to identically treated littermate controls. While there were no significant statistical differences between wildtype and Ly49Q-deficient pDCs in terms of IL6 and TNF-α production, there were clear trends for slightly increased production of cytokine production by Ly49Q-deficient pDCs. Small cohorts of 3 animals per treatment were used due to animal colony and time constraints. These experiments can benefit from larger cohorts of study animals. Even with larger groups of mice, we might speculate that multiple signaling pathways are generated downstream of TLR-9 signaling. Some components may be modulated by Ly49Q (IFN-α), while others may not as evidenced by normal levels of MHC II and CD86. It has been shown that IL6 and TNF-α activation arise from signals received downstream of aggregation in late endosomes (78).

We observed that Ly49Q-deficient mice displayed a severe defect in IFN-α production in addition to a consistent and statistically significant increase in MCMV susceptibility. While the IFN-α defect was seen with overnight pDC culture *in vitro*, this was not the case in serum IFN-α obtained from MCMV challenged mice bled over a time course of 0 to 48 hours at 12 hour intervals. It is very likely that cells other than pDCs are contributing to the anti-viral MCMV

response, especially at later time points (123, 136). Therefore, it would be imperative to examine serum IFN-α at earlier time points on an expanded scale (between 0 to 12 hours at 2 hour intervals). *In vitro* IFN-α responses by pDC incubated with MCMV (Chapter 3, Figure 8A) *and in vivo* responses by CpG (Chapter 3, Figure 4G) were much lower than *in vivo* MCMV responses at 36 hours (Chapter 3, Figure 4C). These lower levels of early IFN-α may be more important in containing viral infection and initiating adaptive immune responses than later IFN-α production.

We previously stated that the defect in IFN-α production observed in our Ly49Q-deficient mice agrees with the study conducted by Omatsu et al. (111). They found that Ly49Q-negative BM pDCs produced significantly less type I IFN that their Ly49Q-positive counterparts when cultured in the present of CpG or SeV. We, however, did not agree with the study performed by Kamogawa-Schifter et al. (243) where they observed comparable levels of IFN-α production from in vitro cultured pDCs stimulated with CpG. We originally questioned the differences in study results from these two studies, which were conducted following parallel experimental setup. However, in retrospect, there were indeed many important differences in these studies. Omatsu et al. isolated *ex vivo* cells directly from bone marrow and measure IFN activity by bioassay (111). Kamogawa-Schifter et al. isolated pDC from Flt3L bone marrow cultures and measured IFN-α by ELISA (243). Plasmacytoid DCs obtained from Flt3L bone marrow cultures are thought to be more activated than their naïve counter parts extracted from bone marrow (28). These activated pDCs have been shown to

secrete less IFN-α compared to pDC from primary lymphoid organs (28). Thus, the discrepancy in IFN-α production may reflect differences in the developmental stages of the pDCs examined.

In terms of the functional significance of *cis* vs. *trans* Ly49Q interactions, it would be difficult to monitor them *in vivo*. CpG stimulated $H2K^bD^b$-deficient pDCs make less IFN-α in the presence of CpG than wildtype pDC as depicted in Chapter 3, Figure 4G. These results are consistent with the hypothesis that Ly49Q:MHCI interactions are necessary for cytokine production in pDCs. However, these results were obtained in the presence of additional stimuli (CpG). Taking this into consideration, one would assume that Ly49Q is interacting with $H\text{-}2K^b$ in *cis* (since there is less tetramer staining in the wildtype) to produce more IFN-α in the presence of CpG. In the absence of CpG, crosslinking of Ly49Q alone did not result in IFN-α. It would appear that in the presence of CpG, *cis* interactions prime pDCs through Ly49Q. Recent work from Held's group on the concept of Ly49:MHC *cis* interactions demonstrate that *cis* interactions continuously sequester the inhibitory receptor away from the immunological synapse for $H\text{-}2D^d$ interactions in trans. This increases the cell's sensitivity to activating signals (227). One could hypothesize that in the presence of CpG, Ly49Q receptors are interacting with $H\text{-}2K^b$ in *cis*, this sequesters the ITIM containing "inhibitory" Ly49Q away from the immunological synapse where it can interact with H-2Kb in *trans*. This decrease in negative signaling renders pDCs more susceptible to activating signals through pDC receptors such as PDC-TREM (activating receptor) to enhance IFN-α. However, this hypothesis

speculating on the role of *cis* clustering in explaining Ly49Q function may not be the case, as reconstitution of Ly49Q-deficient mice with transgenic Ly49Q mutated at the ITIM and subsequent abrogation of IFN-α suggests that ITIM signaling may be important in Ly49Q-dependent IFN-α production (Figure 1).

Our findings and those of Toyama-Sorimachi's group are notable and unexpected. Based on our knowledge of such receptors in lymphoid cells, it is counterintuitive for the ligation of an ITIM-containing receptor to result in increased cytokine secretion as evidenced by increased IFN-α production with anti-Ly49Q cross-linked pDCs and dampened IFN-α production in the Ly49Q-deficient mice. On the other hand, a survey of mouse and human pDC literature reveals numerous inhibitory ITAM associated receptors (33, 46-49, 89). Cross-linking of human BDCA-2 (89, 90), BDCA-4 (48), NKp44 (49), ILT7 (47) and mouse Siglec-H (33) in combination with CpG or virus all appear to inhibit the ability of pDCs to produce IFN. Therefore, the ITIM-containing Ly49Q may perhaps act as an activating receptor in promoting secretion of type I IFN on pDCs in WT mice. Plasmacytoid DCs are crucial in type I IFN secretion in the initial stages of microbial invasion and MHC class I molecules are upregulated during an inflammatory response. Therefore, it is conceivable that pDC Ly49Q sensing of self MHC class I and copious IFN-α production might be appropriate during the early stages of infection and inflammation, when pDC activation is needed. Consequently, absence of the receptor in Ly49Q-deficient mice results in lower cytokine production and higher viral loads as revealed by our results. The "activating" receptor status of Ly49Q is supported by a generalized increase in

protein phosphorylation after Ly49Q cross-linking, despite recruitment of SHP-1 and SHP-2 to the receptor (35). In addition, the report of morphological changes, as observed after Ly49Q cross-linking (35), usually characterize cellular activation rather than cell inhibition.

Advances in understanding disease mechanisms are hastened by the use of effective animal models of disease. However, in order to be truly valuable, a gene knock-out model needs to respond to experimental challenge at comparable levels to wildtype animals once the deficient gene has been reconstituted. Ly49Q-deficient mice were used to generate much of our *in vivo* and *in vitro* data. In an effort to validate our Ly49Q-deficient mouse model and defective IFN-α phenotype, we obtained Ly49Q transgenic (Tg) mice containing the wildtype Ly49Q gene (Ly49Q-TgWT) under the control of the β-actin promoter (88). Ly49Q-TgWT mice were then crossed with Ly49Q-deficient mice to generate Ly49QKO-Ly49QTgWT mice. Serum IFN-α levels 6 hours post CpG-Dotap injection was assessed by IFN-α sandwich ELISA as previously described (271). Preliminary data demonstrates that Ly49QKO-Ly49QTgWT mice displayed high levels of serum IFN-α production compared to Ly49Q-deficient mice, almost reaching the same IFN-α production levels as Ly49Q WT mice (Figure 1). This data suggests that placing the Ly49Q-TgWT transgene back into the Ly49Q-deficienct mice effectively recovered the IFN-α defect in response to CpG and confirms the positive regulatory role of Ly49Q in pDC IFN-α production. The recovery of the IFN-α defect in Ly49Q-deficient mice reconstituted with the WT Ly49Q transgene brings up an important question regarding whether pDCs can

function normally in the absence of Ly49Q. Our *in vitro* data demonstrates that WT pDCs from 129 strain mice secrete large amounts of IFN-α in response to CpG. However, upon blocking with anti-Ly49Q mAb, pDC IFN-α production is significantly reduced (271). This ability of soluble anti-Ly49Q blocking mAb to induce and inhibit IFN-α production suggests that the cells are not truly anergic. In addition, maturation levels of Ly49Q-deficient pDCs and production of other pDC cytokines (TNF-α, IL6) are normal compared to wildtype littermates (271).

The next logical step in our efforts to characterize the Ly49Q receptor involves elucidating the signaling molecules downstream of Ly49Q-H-2K^b ligation on pDCs specifically. All previous studies conducted to characterize Ly49Q receptor signaling involved transfection of Ly49Q into tumor cell lines and non-pDCs that do not physiologically express Ly49Q (35, 88). Analysis of the signaling components activated downstream of Ly49Q ligation within these cell lines presumes that pDCs signal in a parallel fashion. Plasmacytoid DCs uniquely straddle the innate and adaptive branches of the immune system and therefore it is quite likely that they harbor signaling proteins that do not necessarily mirror other lymphoid cells. To dissect Ly49Q signaling mechanisms, we have obtained another Ly49Q transgenic mouse line (88) containing a phenylalanine substitution for tyrosine in the ITIM motif of the Ly49Q transmembrane domain. These mice were designated Ly49Q-TgYF mice and were crossed with Ly49Q-deficient mice to obtain Ly49QKO-Ly49QTgYF mice. Serum IFN-α levels 6 hours following CpG-Dotap injection was then assessed by IFN-α sandwich ELISA. Preliminary unpublished data suggest that Ly49QKO-

Ly49QTgYF pDCs displayed comparable low levels of IFN-α in response to CpG compared to Ly49Q-deficient mice. This data shows that the Ly49Q-TgYF transgene does not rescue IFN-α secretion and supports an ITIM-dependent pathway downstream of Ly49Q activation (Figure 1).

It has been shown that both SHP-1 and -2 are recruited to the Ly49Q ITIM upon treatment of Ly49Q transfected COS7 cells with pervanadate (35). However whether these phosphatases are actually present and recruited in pDC remains unknown. Efforts are currently ongoing to use *ex vivo* sorted pDC to immunoblot for the presence of SHP-1, -2 and SHIP. We carried out a preliminary screen for these phosphatases and all three were present in the immunoblot of pDC whole cell lysate. Once the presence of the phosphatases in whole cell lysates have been established, immunoprecipitation of the phosphatase with anti-Ly49Q cross-linking will be performed to detect specific recruitment of phosphatase to the cytoplasmic ITIM of Ly49Q. These preliminary biochemical approaches will set the stage for characterizing proximal and distal signaling components downstream of Ly49Q activation.

Identifying the signaling molecules downstream of Ly49Q:MHCI interaction can help us reconcile the activating function of Ly49Q in pDCs with the inhibitory function of ITIM-containing Ly49 receptors in observed in NK cells. In an effort to align Ly49Q signaling into known pDC literature, we propose that Ly49Q:MHC-I interactions recruits SHP-1/-2 and this phosphatase antagonizes inhibitory DAP-12 signal transduction. This results in increased IFNa production. Another possibility is that downstream Ly49Q signaling

molecules may directly regulate the TLR9 signaling cascade independently of DAP-12 function. Noriko et al. demonstrated that Ly49Q:H2Kb co-localization is internalized in acidic vesicles and these vesicles further co-localize with CpG containing endo/lysosomes in pDCs and peritoneal exudates macrophages from $Ly49Q^{+/-}$ mice. However, in pDCs and macrophages cells lacking Ly49Q, co-localization did not occur and CpG trafficking structures were impaired (88). These data suggests that Ly49Q maybe be involved in the temporal-spatioregulation CpG and TLR-9 containing endosomes for optimal cytokine production. A third mechanism behind Ly49Q function may include an alternative signaling pathway found in pDC, one which is distinct from canonical inhibitory Ly49 signaling in NK cells and does not involve the recruitment of tyrosine phosphatases. Evidence for this possibility includes Ly49Q-transfected macrophage cell lines, which show an increase in tyrosine-phosporylated proteins in whole cell lysates after mAb-mediated crosslinking of Ly49Q (35). Refer to Chapter 4, Figure 2 for schematic on proposed model of Ly49Q signaling.

Biochemical analyses of pDC receptors are laborious and difficult. Isolation of pDCs by positive or negative techniques by either magnetic or fluorescent cell sorting can yield high purity cells, but in limiting numbers. In addition, positive selection of pDCs using anti-pDC receptor magnetic beads compromises their function (unpublished observations) and should be avoided if isolated cells are to be further activated for functional analyses. In mice, many investigators use pDCs that are derived from bone marrow cultured with Flt3L, which results in a greater pDC yield. The problem with these studies, however, is that *in vitro* generated pDCs do not necessarily represent physiological pDCs in

phenotype or function as well as *ex vivo* sorted splenic pDCs. We exclusively used *ex vivo* pDCs isolated by positive selection of mPDCA-1^{+} cells. While, these pDCs are more physiologically relevant that Flt3L generated pDC cultures, they contain 10-15% contaminating lymphoid cell populations, likely B cells that upregulate mPDCA-1 expression upon activation (IFN-α, IFN-γ). B cells also express TLR-9 and may play a role in CpG recognition. To exclude the possibility of B cell contamination and contribution in our pDC cultures, we sorted CD19^{+} B cells from whole spleen in wildtype and Ly49Q-deficient mice and subjected them to the same experimental set up as our pDCs for CpG stimulation. Only negligible amounts of supernatant IFN-α were detected.

Related to the above issues of experimental limitations, the synthetic agonists and viral model used in our studies brings into question their physiological relevance and limitations. We examined Ly49Q function on pDCs in a very limited fashion. We studied pDC function in isolation with in vitro CpG and virus incubation. Animal and human pDCs do not exist in isolation *in vivo* and many cell types contribute to type I IFN production in the face of microbial challenge. Plasmacytoid DC-derived IFN-α contribution to viral challenge may be important in early response and less important at later time points when other cells also secrete type I IFN. Animals and humans are also unlikely to be naturally infected with 600 to 6000 PFU of virus, although we obtained our MCMV stocks from mice salivary glands. Since MCMV naturally exists in rodent salivary glands and is passed on to non-infected animals through biting and direct transmission into blood vessels (272), "natural" infectious viral titres

passed on in the chain of infection likely reaches 600 to 6000 PFU. Therefore, there is some level of physiological relevance to our viral model. The function of Ly49Q in wild mice and in the context of natural infections remains unknown. The results conducted and discussed in this thesis represent basic immunology research. Translational research attempts should be made to search for the human functional analogue to mouse Ly49Q. Candidate receptors on human pDCs should be examined for their involvement in IFN-α modulation to approach future therapeutic use of Ly49Q.

At present, no such NK receptor family member expressed on human pDCs that recognizes HLA exists. Although a few human pDC potential candidates do exist. DCIR is one such receptor, but it does not belong to an NK receptor family. It contains a cytoplasmic ITIM and has been found to inhibit type I IFN production upon antibody mediated crosslinking. However, the physiological ligand of DCIR is unknown and the interaction between receptor:endogenous ligand may lead to differential downstream signaling (50). CD300a/c are two related members of the Ig-superfamily expressed on leukocytes, including NK cells and pDCs. CD300a contains three ITIMs, while CD300c contains no obvious signaling motifs and probably signals through an unidentified ITAM-containing adaptor. Antibody crosslinking (crossreactivity with both CD300a/c) results in enhanced IFNa, but suppresses TNFa and IL6 production by pDCs in response to CpG-ODN. The endogenous ligands are unknown and it is not yet clear whether these effects are due to ITIM or ITAM signaling (273).

Thorough understanding of the functions of innate receptors such as those that reside on pDCs are important research questions with broad implications. Through their production of important cytokines, as well as through cell-contact dependent mechanisms, pDCs have the ability to interact with multiple components of the innate and adaptive immune systems resulting in either dampened or exaggerated responses. The individual contribution of each pDC receptor to pathogens is an important aspect of the comprehension of pDC function. Because murine pDCs share many morphological and functional characteristics with human pDCs, insight into human pDC immune response against transformed and pathogen infected cells can be acquired from characterizing the Ly49Q ligand and receptor in the mouse model.

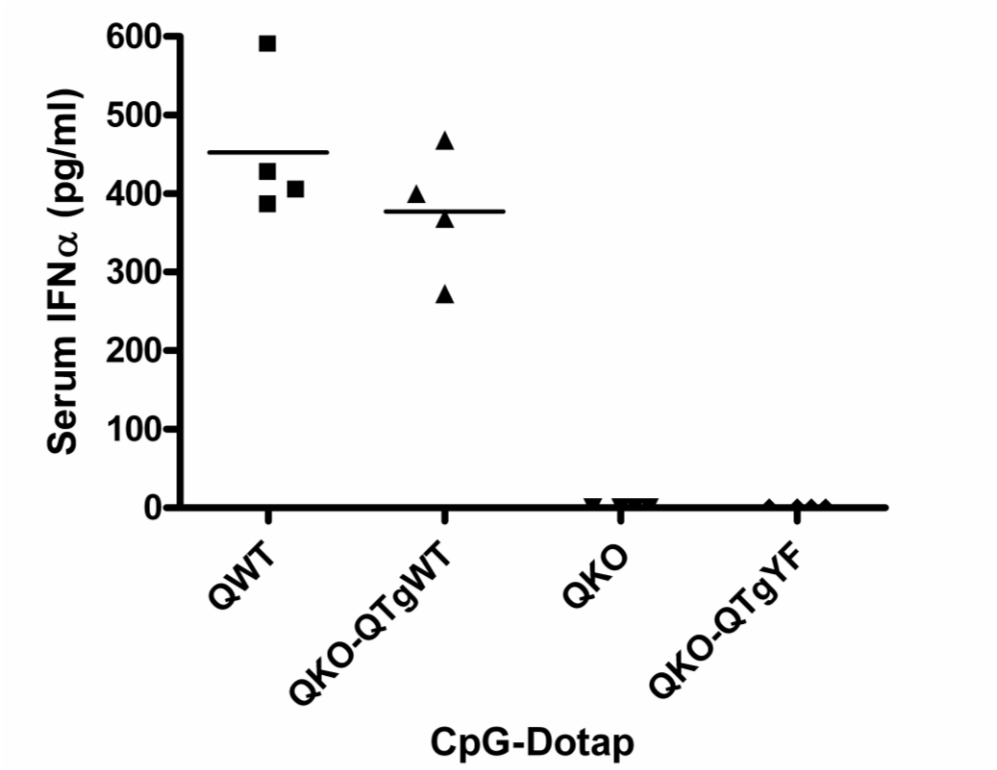

Figure 1. Ly49Q-deficient mice reconstituted with Ly49Q WT and YF transgene. Ly49Q WT transgenic mice and Ly49Q YF transgenic mice (ITIM mutation at phosphotyrosine) are crossed to Ly49Q-deficient mice to generate Ly49QKO-Ly49QTgWT or Ly49QKO-Ly49QTgYF. Ly49Q WT, Ly49QKO-Ly49QTgWT, Ly49Q KO and Ly49QKO-Ly49QTgYF mice were injected with CpG-ODN + DOTAP and blood samples were taken after 6h for serum IFN-α ELISA from four mice of each genotype. Data are representative of experiments done at least three times independently.

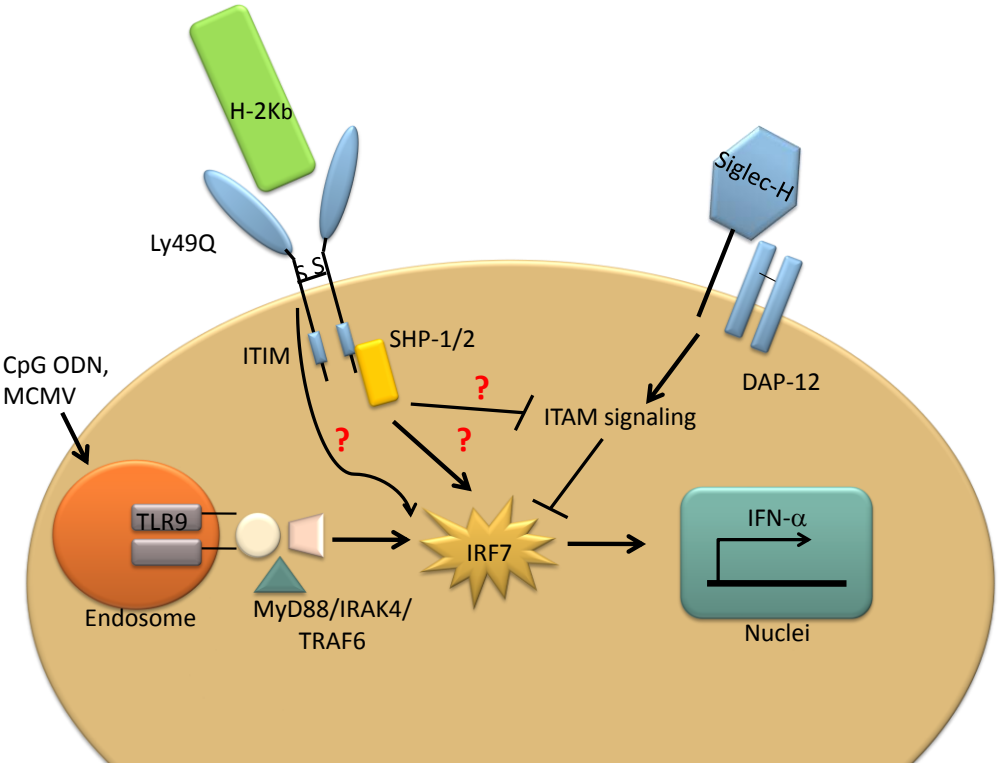

Figure 2. Proposed model for Ly49Q signaling. We propose that Ly49Q:MHC-I interactions recruits SHP-1/-2 and this phosphatase antagonizes inhibitory DAP-12 signal transduction (ex: coupled to mouse SiglecH). This results in increased IFN-α production. Another possibility is that downstream Ly49Q signaling molecules may directly regulate the TLR-9 signaling cascade independently of DAP-12 function. Ly49Q:H-2K^{b} interactions maybe be involved in the temporal-spatioregulation CpG and TLR-9 containing endosomes for optimal cytokine production. A third mechanism behind Ly49Q function may include an alternative signaling pathway found in pDC, one which is distinct from canonical inhibitory Ly49 signaling in NK cells and does not involve the recruitment of tyrosine phosphatases.

REFERENCES

1. Isaacs, A., and J. Lindenmann. 1957. Virus interference. I. The interferon. *Proceedings of the Royal Society of London. Series B, Containing papers of a Biological character* 147:258-267.
2. Trinchieri, G., and D. Santoli. 1978. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. *The Journal of experimental medicine* 147:1314-1333.
3. Saksela, E., I. Virtanen, T. Hovi, D.S. Secher, and K. Cantell. 1984. Monocyte is the main producer of human leukocyte alpha interferons following Sendai virus induction. *Prog Med Virol* 30:78-86.
4. Perussia, B., V. Fanning, and G. Trinchieri. 1985. A leukocyte subset bearing HLA-DR antigens is responsible for in vitro alpha interferon production in response to viruses. *Nat Immun Cell Growth Regul* 4:120-137.
5. Perussia, B., D. Santoli, and G. Trinchieri. 1980. Interferon modulation of natural killer cell activity. *Ann N Y Acad Sci* 350:55-62.
6. Fitzgerald-Bocarsly, P., M. Feldman, M. Mendelsohn, S. Curl, and C. Lopez. 1988. Human mononuclear cells which produce interferon-alpha during NK(HSV-FS) assays are HLA-DR positive cells distinct from cytolytic natural killer effectors. *Journal of leukocyte biology* 43:323-334.
7. Ronnblom, L., U. Ramstedt, and G.V. Alm. 1983. Properties of human natural interferon-producing cells stimulated by tumor cell lines. *European journal of immunology* 13:471-476.
8. Ferbas, J.J., J.F. Toso, A.J. Logar, J.S. Navratil, and C.R. Rinaldo, Jr. 1994. CD4+ blood dendritic cells are potent producers of IFN-alpha in response to in vitro HIV-1 infection. *J Immunol* 152:4649-4662.
9. Feldman, M., and P. Fitzgerald-Bocarsly. 1990. Sequential enrichment and immunocytochemical visualization of human interferon-alpha-producing cells. *J Interferon Res* 10:435-446.
10. Lennert, K., and W. Remmele. 1958. [Karyometric research on lymph node cells in man. I. Germinoblasts, lymphoblasts & lymphocytes.]. *Acta Haematol* 19:99-113.
11. Grouard, G., M.C. Rissoan, L. Filgueira, I. Durand, J. Banchereau, and Y.J. Liu. 1997. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *The Journal of experimental medicine* 185:1101-1111.
12. Cella, M., D. Jarrossay, F. Facchetti, O. Alebardi, H. Nakajima, A. Lanzavecchia, and M. Colonna. 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nature medicine* 5:919-923.
13. Nakano, H., M. Yanagita, and M.D. Gunn. 2001. CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *The Journal of experimental medicine* 194:1171-1178.

14. Asselin-Paturel, C., A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Briere, and G. Trinchieri. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nature immunology* 2:1144-1150.
15. Bjorck, P. 2001. Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice. *Blood* 98:3520-3526.
16. Hoshino, K., T. Sugiyama, M. Matsumoto, T. Tanaka, M. Saito, H. Hemmi, O. Ohara, S. Akira, and T. Kaisho. 2006. IkappaB kinase-alpha is critical for interferon-alpha production induced by Toll-like receptors 7 and 9. *Nature* 440:949-953.
17. Manfra, D.J., S.C. Chen, K.K. Jensen, J.S. Fine, M.T. Wiekowski, and S.A. Lira. 2003. Conditional expression of murine Flt3 ligand leads to expansion of multiple dendritic cell subsets in peripheral blood and tissues of transgenic mice. *J Immunol* 170:2843-2852.
18. Pulendran, B., J. Banchereau, S. Burkeholder, E. Kraus, E. Guinet, C. Chalouni, D. Caron, C. Maliszewski, J. Davoust, J. Fay, and K. Palucka. 2000. Flt3-ligand and granulocyte colony-stimulating factor mobilize distinct human dendritic cell subsets in vivo. *J Immunol* 165:566-572.
19. Gilliet, M., A. Boonstra, C. Paturel, S. Antonenko, X.L. Xu, G. Trinchieri, A. O'Garra, and Y.J. Liu. 2002. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *The Journal of experimental medicine* 195:953-958.
20. Brawand, P., D.R. Fitzpatrick, B.W. Greenfield, K. Brasel, C.R. Maliszewski, and T. De Smedt. 2002. Murine plasmacytoid pre-dendritic cells generated from Flt3 ligand-supplemented bone marrow cultures are immature APCs. *J Immunol* 169:6711-6719.
21. Olweus, J., A. BitMansour, R. Warnke, P.A. Thompson, J. Carballido, L.J. Picker, and F. Lund-Johansen. 1997. Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin. *Proceedings of the National Academy of Sciences of the United States of America* 94:12551-12556.
22. Corcoran, L., I. Ferrero, D. Vremec, K. Lucas, J. Waithman, M. O'Keeffe, L. Wu, A. Wilson, and K. Shortman. 2003. The lymphoid past of mouse plasmacytoid cells and thymic dendritic cells. *J Immunol* 170:4926-4932.
23. Spits, H., F. Couwenberg, A.Q. Bakker, K. Weijer, and C.H. Uittenbogaart. 2000. Id2 and Id3 inhibit development of CD34(+) stem cells into predendritic cell (pre-DC)2 but not into pre-DC1. Evidence for a lymphoid origin of pre-DC2. *The Journal of experimental medicine* 192:1775-1784.
24. Shigematsu, H., B. Reizis, H. Iwasaki, S. Mizuno, D. Hu, D. Traver, P. Leder, N. Sakaguchi, and K. Akashi. 2004. Plasmacytoid dendritic cells activate lymphoid-specific genetic programs irrespective of their cellular origin. *Immunity* 21:43-53.
25. Zuniga, E.I., D.B. McGavern, J.L. Pruneda-Paz, C. Teng, and M.B. Oldstone. 2004. Bone marrow plasmacytoid dendritic cells can

differentiate into myeloid dendritic cells upon virus infection. *Nature immunology* 5:1227-1234.

26. Tamura, T., P. Tailor, K. Yamaoka, H.J. Kong, H. Tsujimura, J.J. O'Shea, H. Singh, and K. Ozato. 2005. IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. *J Immunol* 174:2573-2581.
27. Allman, D., M. Dalod, C. Asselin-Paturel, T. Delale, S.H. Robbins, G. Trinchieri, C.A. Biron, P. Kastner, and S. Chan. 2006. Ikaros is required for plasmacytoid dendritic cell differentiation. *Blood* 108:4025-4034.
28. Reizis, B. Regulation of plasmacytoid dendritic cell development. *Current opinion in immunology* 22:206-211.
29. Fitzgerald-Bocarsly, P., J. Dai, and S. Singh. 2008. Plasmacytoid dendritic cells and type I IFN: 50 years of convergent history. *Cytokine & growth factor reviews* 19:3-19.
30. O'Keeffe, M., H. Hochrein, D. Vremec, I. Caminschi, J.L. Miller, E.M. Anders, L. Wu, M.H. Lahoud, S. Henri, B. Scott, P. Hertzog, L. Tatarczuch, and K. Shortman. 2002. Mouse plasmacytoid cells: long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8(+) dendritic cells only after microbial stimulus. *The Journal of experimental medicine* 196:1307-1319.
31. Blasius, A.L., E. Giurisato, M. Cella, R.D. Schreiber, A.S. Shaw, and M. Colonna. 2006. Bone marrow stromal cell antigen 2 is a specific marker of type I IFN-producing cells in the naive mouse, but a promiscuous cell surface antigen following IFN stimulation. *J Immunol* 177:3260-3265.
32. Asselin-Paturel, C., G. Brizard, J.J. Pin, F. Briere, and G. Trinchieri. 2003. Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody. *J Immunol* 171:6466-6477.
33. Blasius, A.L., M. Cella, J. Maldonado, T. Takai, and M. Colonna. 2006. Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. *Blood* 107:2474-2476.
34. Watarai, H., E. Sekine, S. Inoue, R. Nakagawa, T. Kaisho, and M. Taniguchi. 2008. PDC-TREM, a plasmacytoid dendritic cell-specific receptor, is responsible for augmented production of type I interferon. *Proceedings of the National Academy of Sciences of the United States of America* 105:2993-2998.
35. Toyama-Sorimachi, N., Y. Tsujimura, M. Maruya, A. Onoda, T. Kubota, S. Koyasu, K. Inaba, and H. Karasuyama. 2004. Ly49Q, a member of the Ly49 family that is selectively expressed on myeloid lineage cells and involved in regulation of cytoskeletal architecture. *Proceedings of the National Academy of Sciences of the United States of America* 101:1016-1021.
36. Shortman, K., and Y.J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nature reviews* 2:151-161.
37. Coquerelle, C., and M. Moser. DC subsets in positive and negative regulation of immunity. *Immunological reviews* 234:317-334.

38. Belz, G.T., G.M. Behrens, C.M. Smith, J.F. Miller, C. Jones, K. Lejon, C.G. Fathman, S.N. Mueller, K. Shortman, F.R. Carbone, and W.R. Heath. 2002. The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. *The Journal of experimental medicine* 196:1099-1104.
39. Gilliet, M., and Y.J. Liu. 2002. Human plasmacytoid-derived dendritic cells and the induction of T-regulatory cells. *Human immunology* 63:1149-1155.
40. Izon, D., K. Rudd, W. DeMuth, W.S. Pear, C. Clendenin, R.C. Lindsley, and D. Allman. 2001. A common pathway for dendritic cell and early B cell development. *J Immunol* 167:1387-1392.
41. Ito, T., Y.J. Liu, and N. Kadowaki. 2005. Functional diversity and plasticity of human dendritic cell subsets. *International journal of hematology* 81:188-196.
42. Heath, W.R., G.T. Belz, G.M. Behrens, C.M. Smith, S.P. Forehan, I.A. Parish, G.M. Davey, N.S. Wilson, F.R. Carbone, and J.A. Villadangos. 2004. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunological reviews* 199:9-26.
43. Colonna, M., G. Trinchieri, and Y.J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nature immunology* 5:1219-1226.
44. Asselin-Paturel, C., and G. Trinchieri. 2005. Production of type I interferons: plasmacytoid dendritic cells and beyond. *The Journal of experimental medicine* 202:461-465.
45. Swiecki, M., and M. Colonna. Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. *Immunological reviews* 234:142-162.
46. Cao, W., D.B. Rosen, T. Ito, L. Bover, M. Bao, G. Watanabe, Z. Yao, L. Zhang, L.L. Lanier, and Y.J. Liu. 2006. Plasmacytoid dendritic cell-specific receptor ILT7-Fc epsilonRI gamma inhibits Toll-like receptor-induced interferon production. *The Journal of experimental medicine* 203:1399-1405.
47. Cao, W., L. Bover, M. Cho, X. Wen, S. Hanabuchi, M. Bao, D.B. Rosen, Y.H. Wang, J.L. Shaw, Q. Du, C. Li, N. Arai, Z. Yao, L.L. Lanier, and Y.J. Liu. 2009. Regulation of TLR7/9 responses in plasmacytoid dendritic cells by BST2 and ILT7 receptor interaction. *The Journal of experimental medicine* 206:1603-1614.
48. Grage-Griebenow, E., S. Loseke, M. Kauth, K. Gehlhar, R. Zawatzky, and A. Bufe. 2007. Anti-BDCA-4 (neuropilin-1) antibody can suppress virus-induced IFN-alpha production of plasmacytoid dendritic cells. *Immunology and cell biology* 85:383-390.
49. Fuchs, A., M. Cella, T. Kondo, and M. Colonna. 2005. Paradoxic inhibition of human natural interferon-producing cells by the activating receptor NKp44. *Blood* 106:2076-2082.
50. Meyer-Wentrup, F., D. Benitez-Ribas, P.J. Tacken, C.J. Punt, C.G. Figdor, I.J. de Vries, and G.J. Adema. 2008. Targeting DCIR on human plasmacytoid dendritic cells results in antigen presentation and inhibits IFN-alpha production. *Blood* 111:4245-4253.

51. Bave, U., M. Magnusson, M.L. Eloranta, A. Perers, G.V. Alm, and L. Ronnblom. 2003. Fc gamma RIIa is expressed on natural IFN-alpha-producing cells (plasmacytoid dendritic cells) and is required for the IFN-alpha production induced by apoptotic cells combined with lupus IgG. *J Immunol* 171:3296-3302.
52. Marafioti, T., J.C. Paterson, E. Ballabio, K.K. Reichard, S. Tedoldi, K. Hollowood, M. Dictor, M.L. Hansmann, S.A. Pileri, M.J. Dyer, S. Sozzani, I. Dikic, A.S. Shaw, T. Petrella, H. Stein, P.G. Isaacson, F. Facchetti, and D.Y. Mason. 2008. Novel markers of normal and neoplastic human plasmacytoid dendritic cells. *Blood* 111:3778-3792.
53. Fitzgerald-Bocarsly, P. 2002. Natural interferon-alpha producing cells: the plasmacytoid dendritic cells. *BioTechniques* Suppl:16-20, 22, 24-19.
54. Diebold, S.S. 2009. Activation of dendritic cells by toll-like receptors and C-type lectins. *Handb Exp Pharmacol* 3-30.
55. Penna, G., M. Vulcano, S. Sozzani, and L. Adorini. 2002. Differential migration behavior and chemokine production by myeloid and plasmacytoid dendritic cells. *Human immunology* 63:1164-1171.
56. Yoneyama, H., K. Matsuno, Y. Zhang, T. Nishiwaki, M. Kitabatake, S. Ueha, S. Narumi, S. Morikawa, T. Ezaki, B. Lu, C. Gerard, S. Ishikawa, and K. Matsushima. 2004. Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules. *Int Immunol* 16:915-928.
57. Kohrgruber, N., M. Groger, P. Meraner, E. Kriehuber, P. Petzelbauer, S. Brandt, G. Stingl, A. Rot, and D. Maurer. 2004. Plasmacytoid dendritic cell recruitment by immobilized CXCR3 ligands. *J Immunol* 173:6592-6602.
58. Asselin-Paturel, C., G. Brizard, K. Chemin, A. Boonstra, A. O'Garra, A. Vicari, and G. Trinchieri. 2005. Type I interferon dependence of plasmacytoid dendritic cell activation and migration. *The Journal of experimental medicine* 201:1157-1167.
59. Pascale, F., V. Contreras, M. Bonneau, A. Courbet, S. Chilmonczyk, C. Bevilacqua, M. Epardaud, V. Niborski, S. Riffault, A.M. Balazuc, E. Foulon, L. Guzylack-Piriou, B. Riteau, J. Hope, N. Bertho, B. Charley, and I. Schwartz-Cornil. 2008. Plasmacytoid dendritic cells migrate in afferent skin lymph. *J Immunol* 180:5963-5972.
60. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annual review of immunology* 21:335-376.
61. Takeda, K., and S. Akira. 2003. Toll receptors and pathogen resistance. *Cell Microbiol* 5:143-153.
62. Lemaitre, B., E. Nicolas, L. Michaut, J.M. Reichhart, and J.A. Hoffmann. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86:973-983.
63. Janeway, C.A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annual review of immunology* 20:197-216.

64. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388:394-397.
65. Poltorak, A., X. He, I. Smirnova, M.Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science (New York, N.Y* 282:2085-2088.
66. Hayashi, F., K.D. Smith, A. Ozinsky, T.R. Hawn, E.C. Yi, D.R. Goodlett, J.K. Eng, S. Akira, D.M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410:1099-1103.
67. Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C.J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem* 274:17406-17409.
68. Alexopoulou, L., A.C. Holt, R. Medzhitov, and R.A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413:732-738.
69. Lund, J.M., L. Alexopoulou, A. Sato, M. Karow, N.C. Adams, N.W. Gale, A. Iwasaki, and R.A. Flavell. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proceedings of the National Academy of Sciences of the United States of America* 101:5598-5603.
70. Diebold, S.S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science (New York, N.Y* 303:1529-1531.
71. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740-745.
72. Heil, F., P. Ahmad-Nejad, H. Hemmi, H. Hochrein, F. Ampenberger, T. Gellert, H. Dietrich, G. Lipford, K. Takeda, S. Akira, H. Wagner, and S. Bauer. 2003. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. *European journal of immunology* 33:2987-2997.
73. Akira, S., M. Yamamoto, and K. Takeda. 2003. Role of adapters in Toll-like receptor signalling. *Biochem Soc Trans* 31:637-642.
74. Hazeki, K., N. Masuda, K. Funami, N. Sukenobu, M. Matsumoto, S. Akira, K. Takeda, T. Seya, and O. Hazeki. 2003. Toll-like receptor-mediated tyrosine phosphorylation of paxillin via MyD88-dependent and -independent pathways. *European journal of immunology* 33:740-747.
75. Sato, S., M. Sugiyama, M. Yamamoto, Y. Watanabe, T. Kawai, K. Takeda, and S. Akira. 2003. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. *J Immunol* 171:4304-4310.
76. Roach, J.C., G. Glusman, L. Rowen, A. Kaur, M.K. Purcell, K.D. Smith, L.E. Hood, and A. Aderem. 2005. The evolution of vertebrate Toll-like

receptors. *Proceedings of the National Academy of Sciences of the United States of America* 102:9577-9582.
77. Cao, W., and Y.J. Liu. 2007. Innate immune functions of plasmacytoid dendritic cells. *Current opinion in immunology* 19:24-30.
78. Gilliet, M., W. Cao, and Y.J. Liu. 2008. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nature reviews* 8:594-606.
79. Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science (New York, N.Y* 301:640-643.
80. Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, L. Alexopoulou, R.A. Flavell, and B. Beutler. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proceedings of the National Academy of Sciences of the United States of America* 101:3516-3521.
81. Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda, and S. Akira. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nature immunology* 4:1144-1150.
82. Kawai, T., S. Sato, K.J. Ishii, C. Coban, H. Hemmi, M. Yamamoto, K. Terai, M. Matsuda, J. Inoue, S. Uematsu, O. Takeuchi, and S. Akira. 2004. Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nature immunology* 5:1061-1068.
83. Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 434:772-777.
84. Stetson, D.B., and R. Medzhitov. 2006. Antiviral defense: interferons and beyond. *The Journal of experimental medicine* 203:1837-1841.
85. Lee, H.K., J.M. Lund, B. Ramanathan, N. Mizushima, and A. Iwasaki. 2007. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. *Science (New York, N.Y* 315:1398-1401.
86. Hochrein, H., B. Schlatter, M. O'Keeffe, C. Wagner, F. Schmitz, M. Schiemann, S. Bauer, M. Suter, and H. Wagner. 2004. Herpes simplex virus type-1 induces IFN-alpha production via Toll-like receptor 9-dependent and -independent pathways. *Proceedings of the National Academy of Sciences of the United States of America* 101:11416-11421.
87. Wang, J.P., D.R. Asher, M. Chan, E.A. Kurt-Jones, and R.W. Finberg. 2007. Cutting Edge: Antibody-mediated TLR7-dependent recognition of viral RNA. *J Immunol* 178:3363-3367.
88. Yoshizaki, M., A. Tazawa, E. Kasumi, S. Sasawatari, K. Itoh, T. Dohi, T. Sasazuki, K. Inaba, A.P. Makrigiannis, and N. Toyama-Sorimachi. 2009. Spatiotemporal regulation of intracellular trafficking of Toll-like receptor 9 by an inhibitory receptor, Ly49Q. *Blood* 114:1518-1527.

89. Dzionek, A., Y. Sohma, J. Nagafune, M. Cella, M. Colonna, F. Facchetti, G. Gunther, I. Johnston, A. Lanzavecchia, T. Nagasaka, T. Okada, W. Vermi, G. Winkels, T. Yamamoto, M. Zysk, Y. Yamaguchi, and J. Schmitz. 2001. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *The Journal of experimental medicine* 194:1823-1834.
90. Dzionek, A., Y. Inagaki, K. Okawa, J. Nagafune, J. Rock, Y. Sohma, G. Winkels, M. Zysk, Y. Yamaguchi, and J. Schmitz. 2002. Plasmacytoid dendritic cells: from specific surface markers to specific cellular functions. *Human immunology* 63:1133-1148.
91. Rock, J., E. Schneider, J.R. Grun, A. Grutzkau, R. Kuppers, J. Schmitz, and G. Winkels. 2007. CD303 (BDCA-2) signals in plasmacytoid dendritic cells via a BCR-like signalosome involving Syk, Slp65 and PLCgamma2. *European journal of immunology* 37:3564-3575.
92. Vitale, M., C. Bottino, S. Sivori, L. Sanseverino, R. Castriconi, E. Marcenaro, R. Augugliaro, L. Moretta, and A. Moretta. 1998. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *The Journal of experimental medicine* 187:2065-2072.
93. Sivori, S., S. Parolini, E. Marcenaro, R. Millo, C. Bottino, and A. Moretta. 2000. Triggering receptors involved in natural killer cell-mediated cytotoxicity against choriocarcinoma cell lines. *Human immunology* 61:1055-1058.
94. Lanier, L.L. 2005. NK cell recognition. *Annual review of immunology* 23:225-274.
95. Crocker, P.R., J.C. Paulson, and A. Varki. 2007. Siglecs and their roles in the immune system. *Nature reviews* 7:255-266.
96. Carlin, A.F., Y.C. Chang, T. Areschoug, G. Lindahl, N. Hurtado-Ziola, C.C. King, A. Varki, and V. Nizet. 2009. Group B Streptococcus suppression of phagocyte functions by protein-mediated engagement of human Siglec-5. *The Journal of experimental medicine* 206:1691-1699.
97. Blasius, A.L., and M. Colonna. 2006. Sampling and signaling in plasmacytoid dendritic cells: the potential roles of Siglec-H. *Trends in immunology* 27:255-260.
98. Zhang, J., A. Raper, N. Sugita, R. Hingorani, M. Salio, M.J. Palmowski, V. Cerundolo, and P.R. Crocker. 2006. Characterization of Siglec-H as a novel endocytic receptor expressed on murine plasmacytoid dendritic cell precursors. *Blood* 107:3600-3608.
99. Chen, H., A. Chedotal, Z. He, C.S. Goodman, and M. Tessier-Lavigne. 1997. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. *Neuron* 19:547-559.
100. Wang, J.P., P. Liu, E. Latz, D.T. Golenbock, R.W. Finberg, and D.H. Libraty. 2006. Flavivirus activation of plasmacytoid dendritic cells

delineates key elements of TLR7 signaling beyond endosomal recognition. *J Immunol* 177:7114-7121.

101. Kupzig, S., V. Korolchuk, R. Rollason, A. Sugden, A. Wilde, and G. Banting. 2003. Bst-2/HM1.24 is a raft-associated apical membrane protein with an unusual topology. *Traffic* 4:694-709.
102. Van Damme, N., D. Goff, C. Katsura, R.L. Jorgenson, R. Mitchell, M.C. Johnson, E.B. Stephens, and J. Guatelli. 2008. The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. *Cell Host Microbe* 3:245-252.
103. Ito, T., H. Kanzler, O. Duramad, W. Cao, and Y.J. Liu. 2006. Specialization, kinetics, and repertoire of type 1 interferon responses by human plasmacytoid predendritic cells. *Blood* 107:2423-2431.
104. Coccia, E.M., M. Severa, E. Giacomini, D. Monneron, M.E. Remoli, I. Julkunen, M. Cella, R. Lande, and G. Uze. 2004. Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. *European journal of immunology* 34:796-805.
105. Gibson, S.J., J.M. Lindh, T.R. Riter, R.M. Gleason, L.M. Rogers, A.E. Fuller, J.L. Oesterich, K.B. Gorden, X. Qiu, S.W. McKane, R.J. Noelle, R.L. Miller, R.M. Kedl, P. Fitzgerald-Bocarsly, M.A. Tomai, and J.P. Vasilakos. 2002. Plasmacytoid dendritic cells produce cytokines and mature in response to the TLR7 agonists, imiquimod and resiquimod. *Cellular immunology* 218:74-86.
106. Karlin, S., I. Ladunga, and B.E. Blaisdell. 1994. Heterogeneity of genomes: measures and values. *Proceedings of the National Academy of Sciences of the United States of America* 91:12837-12841.
107. Krug, A., S. Rothenfusser, V. Hornung, B. Jahrsdorfer, S. Blackwell, Z.K. Ballas, S. Endres, A.M. Krieg, and G. Hartmann. 2001. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *European journal of immunology* 31:2154-2163.
108. Kadowaki, N., S. Ho, S. Antonenko, R.W. Malefyt, R.A. Kastelein, F. Bazan, and Y.J. Liu. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *The Journal of experimental medicine* 194:863-869.
109. Kadowaki, N., and Y.J. Liu. 2002. Natural type I interferon-producing cells as a link between innate and adaptive immunity. *Human immunology* 63:1126-1132.
110. Vollmer, J., R. Weeratna, P. Payette, M. Jurk, C. Schetter, M. Laucht, T. Wader, S. Tluk, M. Liu, H.L. Davis, and A.M. Krieg. 2004. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *European journal of immunology* 34:251-262.
111. Omatsu, Y., T. Iyoda, Y. Kimura, A. Maki, M. Ishimori, N. Toyama-Sorimachi, and K. Inaba. 2005. Development of murine plasmacytoid dendritic cells defined by increased expression of an inhibitory NK receptor, Ly49Q. *J Immunol* 174:6657-6662.

112. Izaguirre, A., B.J. Barnes, S. Amrute, W.S. Yeow, N. Megjugorac, J. Dai, D. Feng, E. Chung, P.M. Pitha, and P. Fitzgerald-Bocarsly. 2003. Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells. *Journal of leukocyte biology* 74:1125-1138.
113. Dai, J., N.J. Megjugorac, S.B. Amrute, and P. Fitzgerald-Bocarsly. 2004. Regulation of IFN regulatory factor-7 and IFN-alpha production by enveloped virus and lipopolysaccharide in human plasmacytoid dendritic cells. *J Immunol* 173:1535-1548.
114. Zucchini, N., G. Bessou, S.H. Robbins, L. Chasson, A. Raper, P.R. Crocker, and M. Dalod. 2008. Individual plasmacytoid dendritic cells are major contributors to the production of multiple innate cytokines in an organ-specific manner during viral infection. *Int Immunol* 20:45-56.
115. Poeck, H., M. Wagner, J. Battiany, S. Rothenfusser, D. Wellisch, V. Hornung, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2004. Plasmacytoid dendritic cells, antigen, and CpG-C license human B cells for plasma cell differentiation and immunoglobulin production in the absence of T-cell help. *Blood* 103:3058-3064.
116. Jego, G., A.K. Palucka, J.P. Blanck, C. Chalouni, V. Pascual, and J. Banchereau. 2003. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. *Immunity* 19:225-234.
117. Megjugorac, N.J., H.A. Young, S.B. Amrute, S.L. Olshalsky, and P. Fitzgerald-Bocarsly. 2004. Virally stimulated plasmacytoid dendritic cells produce chemokines and induce migration of T and NK cells. *Journal of leukocyte biology* 75:504-514.
118. Penna, G., M. Vulcano, A. Roncari, F. Facchetti, S. Sozzani, and L. Adorini. 2002. Cutting edge: differential chemokine production by myeloid and plasmacytoid dendritic cells. *J Immunol* 169:6673-6676.
119. Vanbervliet, B., N. Bendriss-Vermare, C. Massacrier, B. Homey, O. de Bouteiller, F. Briere, G. Trinchieri, and C. Caux. 2003. The inducible CXCR3 ligands control plasmacytoid dendritic cell responsiveness to the constitutive chemokine stromal cell-derived factor 1 (SDF-1)/CXCL12. *The Journal of experimental medicine* 198:823-830.
120. Groot, F., T.M. van Capel, M.L. Kapsenberg, B. Berkhout, and E.C. de Jong. 2006. Opposing roles of blood myeloid and plasmacytoid dendritic cells in HIV-1 infection of T cells: transmission facilitation versus replication inhibition. *Blood* 108:1957-1964.
121. Ryan, L.K., G. Diamond, S. Amrute, Z. Feng, A. Weinberg, and P. Fitzgerald-Bocarsly. 2003. Detection of HBD1 peptide in peripheral blood mononuclear cell subpopulations by intracellular flow cytometry. *Peptides* 24:1785-1794.
122. Ito, T., Y.H. Wang, and Y.J. Liu. 2005. Plasmacytoid dendritic cell precursors/type I interferon-producing cells sense viral infection by Toll-like receptor (TLR) 7 and TLR9. *Springer seminars in immunopathology* 26:221-229.

123. Delale, T., A. Paquin, C. Asselin-Paturel, M. Dalod, G. Brizard, E.E. Bates, P. Kastner, S. Chan, S. Akira, A. Vicari, C.A. Biron, G. Trinchieri, and F. Briere. 2005. MyD88-dependent and -independent murine cytomegalovirus sensing for IFN-alpha release and initiation of immune responses in vivo. *J Immunol* 175:6723-6732.
124. Honda, K., Y. Ohba, H. Yanai, H. Negishi, T. Mizutani, A. Takaoka, C. Taya, and T. Taniguchi. 2005. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature* 434:1035-1040.
125. Barnes, B.J., A.E. Field, and P.M. Pitha-Rowe. 2003. Virus-induced heterodimer formation between IRF-5 and IRF-7 modulates assembly of the IFNA enhanceosome in vivo and transcriptional activity of IFNA genes. *J Biol Chem* 278:16630-16641.
126. Shinohara, M.L., L. Lu, J. Bu, M.B. Werneck, K.S. Kobayashi, L.H. Glimcher, and H. Cantor. 2006. Osteopontin expression is essential for interferon-alpha production by plasmacytoid dendritic cells. *Nature immunology* 7:498-506.
127. Xanthou, G., T. Alissafi, M. Semitekolou, D.C. Simoes, E. Economidou, M. Gaga, B.N. Lambrecht, C.M. Lloyd, and V. Panoutsakopoulou. 2007. Osteopontin has a crucial role in allergic airway disease through regulation of dendritic cell subsets. *Nature medicine* 13:570-578.
128. Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K.K. Conzelmann, M. Schlee, S. Endres, and G. Hartmann. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. *Science (New York, N.Y* 314:994-997.
129. Pichlmair, A., and C. Reis e Sousa. 2007. Innate recognition of viruses. *Immunity* 27:370-383.
130. Sun, Q., L. Sun, H.H. Liu, X. Chen, R.B. Seth, J. Forman, and Z.J. Chen. 2006. The specific and essential role of MAVS in antiviral innate immune responses. *Immunity* 24:633-642.
131. Rothenfusser, S., N. Goutagny, G. DiPerna, M. Gong, B.G. Monks, A. Schoenemeyer, M. Yamamoto, S. Akira, and K.A. Fitzgerald. 2005. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J Immunol* 175:5260-5268.
132. Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nature immunology* 5:730-737.
133. Kato, H., S. Sato, M. Yoneyama, M. Yamamoto, S. Uematsu, K. Matsui, T. Tsujimura, K. Takeda, T. Fujita, O. Takeuchi, and S. Akira. 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23:19-28.
134. Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K.J. Ishii, O. Takeuchi, and S. Akira. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nature immunology* 6:981-988.
135. Fitzgerald-Bocarsly, P., and D. Feng. 2007. The role of type I interferon production by dendritic cells in host defense. *Biochimie* 89:843-855.

136. Dalod, M., T. Hamilton, R. Salomon, T.P. Salazar-Mather, S.C. Henry, J.D. Hamilton, and C.A. Biron. 2003. Dendritic cell responses to early murine cytomegalovirus infection: subset functional specialization and differential regulation by interferon alpha/beta. *The Journal of experimental medicine* 197:885-898.
137. Dalod, M., T.P. Salazar-Mather, L. Malmgaard, C. Lewis, C. Asselin-Paturel, F. Briere, G. Trinchieri, and C.A. Biron. 2002. Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. *The Journal of experimental medicine* 195:517-528.
138. Tam, M.A., and M.J. Wick. 2006. Differential expansion, activation and effector functions of conventional and plasmacytoid dendritic cells in mouse tissues transiently infected with Listeria monocytogenes. *Cell Microbiol* 8:1172-1187.
139. Moseman, E.A., X. Liang, A.J. Dawson, A. Panoskaltsis-Mortari, A.M. Krieg, Y.J. Liu, B.R. Blazar, and W. Chen. 2004. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J Immunol* 173:4433-4442.
140. Gerosa, F., B. Baldani-Guerra, C. Nisii, V. Marchesini, G. Carra, and G. Trinchieri. 2002. Reciprocal activating interaction between natural killer cells and dendritic cells. *The Journal of experimental medicine* 195:327-333.
141. Villadangos, J.A., and L. Young. 2008. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity* 29:352-361.
142. Paludan, C., D. Schmid, M. Landthaler, M. Vockerodt, D. Kube, T. Tuschl, and C. Munz. 2005. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science (New York, N.Y* 307:593-596.
143. Blander, J.M. 2007. Coupling Toll-like receptor signaling with phagocytosis: potentiation of antigen presentation. *Trends in immunology* 28:19-25.
144. Blander, J.M., and R. Medzhitov. 2006. Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature* 440:808-812.
145. Boonstra, A., C. Asselin-Paturel, M. Gilliet, C. Crain, G. Trinchieri, Y.J. Liu, and A. O'Garra. 2003. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. *The Journal of experimental medicine* 197:101-109.
146. Colonna, M. 2006. Toll-like receptors and IFN-alpha: partners in autoimmunity. *The Journal of clinical investigation* 116:2319-2322.
147. Fallarino, F., C. Asselin-Paturel, C. Vacca, R. Bianchi, S. Gizzi, M.C. Fioretti, G. Trinchieri, U. Grohmann, and P. Puccetti. 2004. Murine plasmacytoid dendritic cells initiate the immunosuppressive pathway of tryptophan catabolism in response to CD200 receptor engagement. *J Immunol* 173:3748-3754.
148. Salio, M., M.J. Palmowski, A. Atzberger, I.F. Hermans, and V. Cerundolo. 2004. CpG-matured murine plasmacytoid dendritic cells are capable of in

vivo priming of functional CD8 T cell responses to endogenous but not exogenous antigens. *The Journal of experimental medicine* 199:567-579.
149. Schlecht, G., S. Garcia, N. Escriou, A.A. Freitas, C. Leclerc, and G. Dadaglio. 2004. Murine plasmacytoid dendritic cells induce effector/memory CD8+ T-cell responses in vivo after viral stimulation. *Blood* 104:1808-1815.
150. Rothenfusser, S., V. Hornung, M. Ayyoub, S. Britsch, A. Towarowski, A. Krug, A. Sarris, N. Lubenow, D. Speiser, S. Endres, and G. Hartmann. 2004. CpG-A and CpG-B oligonucleotides differentially enhance human peptide-specific primary and memory CD8+ T-cell responses in vitro. *Blood* 103:2162-2169.
151. Sapoznikov, A., J.A. Fischer, T. Zaft, R. Krauthgamer, A. Dzionek, and S. Jung. 2007. Organ-dependent in vivo priming of naive CD4+, but not CD8+, T cells by plasmacytoid dendritic cells. *The Journal of experimental medicine* 204:1923-1933.
152. Le Bon, A., G. Schiavoni, G. D'Agostino, I. Gresser, F. Belardelli, and D.F. Tough. 2001. Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity* 14:461-470.
153. Ciavarra, R.P., L. Taylor, A.R. Greene, N. Yousefieh, D. Horeth, N. van Rooijen, C. Steel, B. Gregory, M. Birkenbach, and M. Sekellick. 2005. Impact of macrophage and dendritic cell subset elimination on antiviral immunity, viral clearance and production of type 1 interferon. *Virology* 342:177-189.
154. Lund, J.M., M.M. Linehan, N. Iijima, and A. Iwasaki. 2006. Cutting Edge: Plasmacytoid dendritic cells provide innate immune protection against mucosal viral infection in situ. *J Immunol* 177:7510-7514.
155. Wang, H., N. Peters, and J. Schwarze. 2006. Plasmacytoid dendritic cells limit viral replication, pulmonary inflammation, and airway hyperresponsiveness in respiratory syncytial virus infection. *J Immunol* 177:6263-6270.
156. Feldman, S., D. Stein, S. Amrute, T. Denny, Z. Garcia, P. Kloser, Y. Sun, N. Megjugorac, and P. Fitzgerald-Bocarsly. 2001. Decreased interferon-alpha production in HIV-infected patients correlates with numerical and functional deficiencies in circulating type 2 dendritic cell precursors. *Clinical immunology (Orlando, Fla* 101:201-210.
157. Sun, P., S. Fernandez, M.A. Marovich, D.R. Palmer, C.M. Celluzzi, K. Boonnak, Z. Liang, H. Subramanian, K.R. Porter, W. Sun, and T.H. Burgess. 2009. Functional characterization of ex vivo blood myeloid and plasmacytoid dendritic cells after infection with dengue virus. *Virology* 383:207-215.
158. Wang, K., X. Fan, Y. Fan, B. Wang, L. Han, and Y. Hou. 2007. Study on the function of circulating plasmacytoid dendritic cells in the immunoactive phase of patients with chronic genotype B and C HBV infection. *J Viral Hepat* 14:276-282.
159. Finke, D., M.L. Eloranta, and L. Ronnblom. 2009. Endogenous type I interferon inducers in autoimmune diseases. *Autoimmunity* 42:349-352.

160. Means, T.K., E. Latz, F. Hayashi, M.R. Murali, D.T. Golenbock, and A.D. Luster. 2005. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. *The Journal of clinical investigation* 115:407-417.
161. Means, T.K., and A.D. Luster. 2005. Toll-like receptor activation in the pathogenesis of systemic lupus erythematosus. *Ann N Y Acad Sci* 1062:242-251.
162. Tian, J., A.M. Avalos, S.Y. Mao, B. Chen, K. Senthil, H. Wu, P. Parroche, S. Drabic, D. Golenbock, C. Sirois, J. Hua, L.L. An, L. Audoly, G. La Rosa, A. Bierhaus, P. Naworth, A. Marshak-Rothstein, M.K. Crow, K.A. Fitzgerald, E. Latz, P.A. Kiener, and A.J. Coyle. 2007. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nature immunology* 8:487-496.
163. Wildenberg, M.E., C.G. van Helden-Meeuwsen, J.P. van de Merwe, H.A. Drexhage, and M.A. Versnel. 2008. Systemic increase in type I interferon activity in Sjogren's syndrome: a putative role for plasmacytoid dendritic cells. *European journal of immunology* 38:2024-2033.
164. Wollenberg, A., M. Wagner, S. Gunther, A. Towarowski, E. Tuma, M. Moderer, S. Rothenfusser, S. Wetzel, S. Endres, and G. Hartmann. 2002. Plasmacytoid dendritic cells: a new cutaneous dendritic cell subset with distinct role in inflammatory skin diseases. *The Journal of investigative dermatology* 119:1096-1102.
165. Nestle, F.O., C. Conrad, A. Tun-Kyi, B. Homey, M. Gombert, O. Boyman, G. Burg, Y.J. Liu, and M. Gilliet. 2005. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *The Journal of experimental medicine* 202:135-143.
166. Bangert, C., J. Friedl, G. Stary, G. Stingl, and T. Kopp. 2003. Immunopathologic features of allergic contact dermatitis in humans: participation of plasmacytoid dendritic cells in the pathogenesis of the disease? *The Journal of investigative dermatology* 121:1409-1418.
167. Chen, J., Z. Tan, H. Liu, Z. Liu, Y. Wu, and J. Li. 2006. Expression of plasmacytoid dendritic cells, IRF-7, IFN-alpha mRNA in the lesions of psoriasis vulgaris. *Journal of Huazhong University of Science and Technology. Medical sciences = Hua zhong ke ji da xue xue bao. Yi xue Ying De wen ban = Huazhong keji daxue xuebao* 26:747-749.
168. Lande, R., J. Gregorio, V. Facchinetti, B. Chatterjee, Y.H. Wang, B. Homey, W. Cao, Y.H. Wang, B. Su, F.O. Nestle, T. Zal, I. Mellman, J.M. Schroder, Y.J. Liu, and M. Gilliet. 2007. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* 449:564-569.
169. Schauber, J., D. Rieger, F. Weiler, J. Wehkamp, M. Eck, K. Fellermann, W. Scheppach, R.L. Gallo, and E.F. Stange. 2006. Heterogeneous expression of human cathelicidin hCAP18/LL-37 in inflammatory bowel diseases. *Eur J Gastroenterol Hepatol* 18:615-621.
170. Lande, R., E. Giacomini, B. Serafini, B. Rosicarelli, G.D. Sebastiani, G. Minisola, U. Tarantino, V. Riccieri, G. Valesini, and E.M. Coccia. 2004. Characterization and recruitment of plasmacytoid dendritic cells in

synovial fluid and tissue of patients with chronic inflammatory arthritis. *J Immunol* 173:2815-2824.
171. Theofilopoulos, A.N., R. Baccala, B. Beutler, and D.H. Kono. 2005. Type I interferons (alpha/beta) in immunity and autoimmunity. *Annual review of immunology* 23:307-336.
172. Ostrand-Rosenberg, S., and P. Sinha. 2009. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* 182:4499-4506.
173. Lou, Y., C. Liu, G.J. Kim, Y.J. Liu, P. Hwu, and G. Wang. 2007. Plasmacytoid dendritic cells synergize with myeloid dendritic cells in the induction of antigen-specific antitumor immune responses. *J Immunol* 178:1534-1541.
174. Hartmann, E., B. Wollenberg, S. Rothenfusser, M. Wagner, D. Wellisch, B. Mack, T. Giese, O. Gires, S. Endres, and G. Hartmann. 2003. Identification and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer. *Cancer Res* 63:6478-6487.
175. Palamara, F., S. Meindl, M. Holcmann, P. Luhrs, G. Stingl, and M. Sibilia. 2004. Identification and characterization of pDC-like cells in normal mouse skin and melanomas treated with imiquimod. *J Immunol* 173:3051-3061.
176. Kim, R., M. Emi, K. Tanabe, and K. Arihiro. 2007. Potential functional role of plasmacytoid dendritic cells in cancer immunity. *Immunology* 121:149-157.
177. Medzhitov, R., and C.A. Janeway, Jr. 2002. Decoding the patterns of self and nonself by the innate immune system. *Science (New York, N.Y* 296:298-300.
178. Lanier, L.L. 1998. NK cell receptors. *Annual review of immunology* 16:359-393.
179. Hamerman, J.A., K. Ogasawara, and L.L. Lanier. 2005. NK cells in innate immunity. *Current opinion in immunology* 17:29-35.
180. Lanier, L.L. 2001. On guard--activating NK cell receptors. *Nature immunology* 2:23-27.
181. Biron, C.A., K.S. Byron, and J.L. Sullivan. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med* 320:1731-1735.
182. Ballas, Z.K., J.M. Turner, D.A. Turner, E.A. Goetzman, and J.D. Kemp. 1990. A patient with simultaneous absence of "classical" natural killer cells (CD3-, CD16+, and NKH1+) and expansion of CD3+, CD4-, CD8-, NKH1+ subset. *J Allergy Clin Immunol* 85:453-459.
183. Joncas, J., Y. Monczak, F. Ghibu, C. Alfieri, A. Bonin, G. Ahronheim, and G. Rivard. 1989. Brief report: killer cell defect and persistent immunological abnormalities in two patients with chronic active Epstein-Barr virus infection. *J Med Virol* 28:110-117.
184. Katz, J.D., R. Mitsuyasu, M.S. Gottlieb, L.T. Lebow, and B. Bonavida. 1987. Mechanism of defective NK cell activity in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. II. Normal antibody-dependent cellular cytotoxicity (ADCC) mediated by

effector cells defective in natural killer (NK) cytotoxicity. *J Immunol* 139:55-60.
185. Natarajan, K., H. Li, R.A. Mariuzza, and D.H. Margulies. 1999. MHC class I molecules, structure and function. *Rev Immunogenet* 1:32-46.
186. Sawicki, M.W., N. Dimasi, K. Natarajan, J. Wang, D.H. Margulies, and R.A. Mariuzza. 2001. Structural basis of MHC class I recognition by natural killer cell receptors. *Immunological reviews* 181:52-65.
187. York, I.A., and K.L. Rock. 1996. Antigen processing and presentation by the class I major histocompatibility complex. *Annual review of immunology* 14:369-396.
188. Rodgers, J.R., and R.G. Cook. 2005. MHC class Ib molecules bridge innate and acquired immunity. *Nature reviews* 5:459-471.
189. Brigl, M., and M.B. Brenner. 2004. CD1: antigen presentation and T cell function. *Annual review of immunology* 22:817-890.
190. Sugita, M., M. Cernadas, and M.B. Brenner. 2004. New insights into pathways for CD1-mediated antigen presentation. *Current opinion in immunology* 16:90-95.
191. Lanier, L.L. 1998. Activating and inhibitory NK cell receptors. *Adv Exp Med Biol* 452:13-18.
192. Lanier, L.L., A.M. Le, S. Cwirla, N. Federspiel, and J.H. Phillips. 1986. Antigenic, functional, and molecular genetic studies of human natural killer cells and cytotoxic T lymphocytes not restricted by the major histocompatibility complex. *Fed Proc* 45:2823-2828.
193. Karre, K., H.G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 319:675-678.
194. Hoglund, P., R. Glas, C. Ohlen, H.G. Ljunggren, and K. Karre. 1991. Alteration of the natural killer repertoire in H-2 transgenic mice: specificity of rapid lymphoma cell clearance determined by the H-2 phenotype of the target. *The Journal of experimental medicine* 174:327-334.
195. Bix, M., N.S. Liao, M. Zijlstra, J. Loring, R. Jaenisch, and D. Raulet. 1991. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature* 349:329-331.
196. Wong, E.S., C.E. Sanderson, J.E. Deakin, C.M. Whittington, A.T. Papenfuss, and K. Belov. 2009. Identification of natural killer cell receptor clusters in the platypus genome reveals an expansion of C-type lectin genes. *Immunogenetics* 61:565-579.
197. Parham, P. 2005. Immunogenetics of killer cell immunoglobulin-like receptors. *Molecular immunology* 42:459-462.
198. Moretta, L., and A. Moretta. 2004. Killer immunoglobulin-like receptors. *Current opinion in immunology* 16:626-633.
199. Kelley, J., L. Walter, and J. Trowsdale. 2005. Comparative genomics of natural killer cell receptor gene clusters. *PLoS Genet* 1:129-139.
200. Carlyle, J.R., A. Mesci, J.H. Fine, P. Chen, S. Belanger, L.H. Tai, and A.P. Makrigiannis. 2008. Evolution of the Ly49 and Nkrp1 recognition systems. *Seminars in immunology* 20:321-330.

201. Carlyle, J.R., A.M. Jamieson, S. Gasser, C.S. Clingan, H. Arase, and D.H. Raulet. 2004. Missing self-recognition of Ocil/Clr-b by inhibitory NKR-P1 natural killer cell receptors. *Proceedings of the National Academy of Sciences of the United States of America* 101:3527-3532.
202. Westgaard, I.H., S.F. Berg, S. Orstavik, S. Fossum, and E. Dissen. 1998. Identification of a human member of the Ly-49 multigene family. *European journal of immunology* 28:1839-1846.
203. Makrigiannis, A.P., and S.K. Anderson. 2003. Regulation of natural killer cell function. *Cancer biology & therapy* 2:610-616.
204. Dimasi, N., and R. Biassoni. 2005. Structural and functional aspects of the Ly49 natural killer cell receptors. *Immunology and cell biology* 83:1-8.
205. Lanier, L.L., B.C. Corliss, J. Wu, C. Leong, and J.H. Phillips. 1998. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature* 391:703-707.
206. Tassi, I., G. Le Friec, S. Gilfillan, T. Takai, W.M. Yokoyama, and M. Colonna. 2009. DAP10 associates with Ly49 receptors but contributes minimally to their expression and function in vivo. *European journal of immunology* 39:1129-1135.
207. Orr, M.T., J.C. Sun, D.G. Hesslein, H. Arase, J.H. Phillips, T. Takai, and L.L. Lanier. 2009. Ly49H signaling through DAP10 is essential for optimal natural killer cell responses to mouse cytomegalovirus infection. *The Journal of experimental medicine* 206:807-817.
208. Lanier, L.L. 2009. DAP10- and DAP12-associated receptors in innate immunity. *Immunological reviews* 227:150-160.
209. Reth, M. 1989. Antigen receptor tail clue. *Nature* 338:383-384.
210. Lee, S.H., S. Girard, D. Macina, M. Busa, A. Zafer, A. Belouchi, P. Gros, and S.M. Vidal. 2001. Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. *Nature genetics* 28:42-45.
211. Nakamura, M.C., P.A. Linnemeyer, E.C. Niemi, L.H. Mason, J.R. Ortaldo, J.C. Ryan, and W.E. Seaman. 1999. Mouse Ly-49D recognizes H-2Dd and activates natural killer cell cytotoxicity. *The Journal of experimental medicine* 189:493-500.
212. George, T.C., L.H. Mason, J.R. Ortaldo, V. Kumar, and M. Bennett. 1999. Positive recognition of MHC class I molecules by the Ly49D receptor of murine NK cells. *J Immunol* 162:2035-2043.
213. George, T.C., J.R. Ortaldo, S. Lemieux, V. Kumar, and M. Bennett. 1999. Tolerance and alloreactivity of the Ly49D subset of murine NK cells. *J Immunol* 163:1859-1867.
214. Silver, E.T., D.E. Gong, C.S. Chang, A. Amrani, P. Santamaria, and K.P. Kane. 2000. Ly-49P activates NK-mediated lysis by recognizing H-2Dd. *J Immunol* 165:1771-1781.
215. Silver, E.T., D. Gong, B. Hazes, and K.P. Kane. 2001. Ly-49W, an activating receptor of nonobese diabetic mice with close homology to the inhibitory receptor Ly-49G, recognizes H-2D(k) and H-2D(d). *J Immunol* 166:2333-2341.

216. Cerwenka, A., and L.L. Lanier. 2001. Ligands for natural killer cell receptors: redundancy or specificity. *Immunological reviews* 181:158-169.
217. Cerwenka, A., and L.L. Lanier. 2001. Natural killer cells, viruses and cancer. *Nature reviews* 1:41-49.
218. McVicar, D.W., and D.N. Burshtyn. 2001. Intracellular signaling by the killer immunoglobulin-like receptors and Ly49. *Sci STKE* 2001:re1.
219. Yu, M.C., L.L. Su, L. Zou, Y. Liu, N. Wu, L. Kong, Z.H. Zhuang, L. Sun, H.P. Liu, J.H. Hu, D. Li, J.L. Strominger, J.W. Zang, G. Pei, and B.X. Ge. 2008. An essential function for beta-arrestin 2 in the inhibitory signaling of natural killer cells. *Nature immunology* 9:898-907.
220. Wang, J., M.C. Whitman, K. Natarajan, J. Tormo, R.A. Mariuzza, and D.H. Margulies. 2002. Binding of the natural killer cell inhibitory receptor Ly49A to its major histocompatibility complex class I ligand. Crucial contacts include both H-2Dd AND beta 2-microglobulin. *J Biol Chem* 277:1433-1442.
221. Tormo, J., K. Natarajan, D.H. Margulies, and R.A. Mariuzza. 1999. Crystal structure of a lectin-like natural killer cell receptor bound to its MHC class I ligand. *Nature* 402:623-631.
222. Dam, J., R. Guan, K. Natarajan, N. Dimasi, L.K. Chlewicki, D.M. Kranz, P. Schuck, D.H. Margulies, and R.A. Mariuzza. 2003. Variable MHC class I engagement by Ly49 natural killer cell receptors demonstrated by the crystal structure of Ly49C bound to H-2K(b). *Nature immunology* 4:1213-1222.
223. Back, J., E.L. Malchiodi, S. Cho, L. Scarpellino, P. Schneider, M.C. Kerzic, R.A. Mariuzza, and W. Held. 2009. Distinct conformations of Ly49 natural killer cell receptors mediate MHC class I recognition in trans and cis. *Immunity* 31:598-608.
224. Scarpellino, L., F. Oeschger, P. Guillaume, J.D. Coudert, F. Levy, G. Leclercq, and W. Held. 2007. Interactions of Ly49 family receptors with MHC class I ligands in trans and cis. *J Immunol* 178:1277-1284.
225. Zimmer, J., V. Ioannidis, and W. Held. 2001. H-2D ligand expression by Ly49A+ natural killer (NK) cells precludes ligand uptake from environmental cells: implications for NK cell function. *The Journal of experimental medicine* 194:1531-1539.
226. Doucey, M.A., L. Scarpellino, J. Zimmer, P. Guillaume, I.F. Luescher, C. Bron, and W. Held. 2004. Cis association of Ly49A with MHC class I restricts natural killer cell inhibition. *Nature immunology* 5:328-336.
227. Back, J., A. Chalifour, L. Scarpellino, and W. Held. 2007. Stable masking by H-2Dd cis ligand limits Ly49A relocalization to the site of NK cell/target cell contact. *Proceedings of the National Academy of Sciences of the United States of America* 104:3978-3983.
228. Hanke, T., H. Takizawa, C.W. McMahon, D.H. Busch, E.G. Pamer, J.D. Miller, J.D. Altman, Y. Liu, D. Cado, F.A. Lemonnier, P.J. Bjorkman, and D.H. Raulet. 1999. Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity* 11:67-77.
229. Michaelsson, J., A. Achour, M. Salcedo, A. Kase-Sjostrom, J. Sundback, R.A. Harris, and K. Karre. 2000. Visualization of inhibitory Ly49 receptor

specificity with soluble major histocompatibility complex class I tetramers. *European journal of immunology* 30:300-307.
230. Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan. 2009. T cell activation. *Annual review of immunology* 27:591-619.
231. Belanger, S., L.H. Tai, S.K. Anderson, and A.P. Makrigiannis. 2008. Ly49 cluster sequence analysis in a mouse model of diabetes: an expanded repertoire of activating receptors in the NOD genome. *Genes and immunity* 9:509-521.
232. Anderson, S.K., K. Dewar, M.L. Goulet, G. Leveque, and A.P. Makrigiannis. 2005. Complete elucidation of a minimal class I MHC natural killer cell receptor haplotype. *Genes and immunity* 6:481-492.
233. Norman, P.J., and P. Parham. 2005. Complex interactions: the immunogenetics of human leukocyte antigen and killer cell immunoglobulin-like receptors. *Semin Hematol* 42:65-75.
234. Arase, H., E.S. Mocarski, A.E. Campbell, A.B. Hill, and L.L. Lanier. 2002. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science (New York, N.Y* 296:1323-1326.
235. Smith, H.R., J.W. Heusel, I.K. Mehta, S. Kim, B.G. Dorner, O.V. Naidenko, K. Iizuka, H. Furukawa, D.L. Beckman, J.T. Pingel, A.A. Scalzo, D.H. Fremont, and W.M. Yokoyama. 2002. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. *Proceedings of the National Academy of Sciences of the United States of America* 99:8826-8831.
236. Makrigiannis, A.P., A.T. Pau, P.L. Schwartzberg, D.W. McVicar, T.W. Beck, and S.K. Anderson. 2002. A BAC contig map of the Ly49 gene cluster in 129 mice reveals extensive differences in gene content relative to C57BL/6 mice. *Genomics* 79:437-444.
237. Proteau, M.F., E. Rousselle, and A.P. Makrigiannis. 2004. Mapping of the BALB/c Ly49 cluster defines a minimal natural killer cell receptor gene repertoire. *Genomics* 84:669-677.
238. Toyama-Sorimachi, N., Y. Omatsu, A. Onoda, Y. Tsujimura, T. Iyoda, A. Kikuchi-Maki, H. Sorimachi, T. Dohi, S. Taki, K. Inaba, and H. Karasuyama. 2005. Inhibitory NK receptor Ly49Q is expressed on subsets of dendritic cells in a cellular maturation- and cytokine stimulation-dependent manner. *J Immunol* 174:4621-4629.
239. Gays, F., J.G. Aust, D.M. Reid, J. Falconer, N. Toyama-Sorimachi, P.R. Taylor, and C.G. Brooks. 2006. Ly49B is expressed on multiple subpopulations of myeloid cells. *J Immunol* 177:5840-5851.
240. Makrigiannis, A.P., D. Patel, M.L. Goulet, K. Dewar, and S.K. Anderson. 2005. Direct sequence comparison of two divergent class I MHC natural killer cell receptor haplotypes. *Genes and immunity* 6:71-83.
241. Liu, Y.J. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annual review of immunology* 23:275-306.
242. Blasius, A., W. Vermi, A. Krug, F. Facchetti, M. Cella, and M. Colonna. 2004. A cell-surface molecule selectively expressed on murine natural

interferon-producing cells that blocks secretion of interferon-alpha. *Blood* 103:4201-4206.
243. Kamogawa-Schifter, Y., J. Ohkawa, S. Namiki, N. Arai, K. Arai, and Y. Liu. 2005. Ly49Q defines 2 pDC subsets in mice. *Blood* 105:2787-2792.
244. Anderson, S.K., J.R. Ortaldo, and D.W. McVicar. 2001. The ever-expanding Ly49 gene family: repertoire and signaling. *Immunological reviews* 181:79-89.
245. Naper, C., K.Z. Dai, L. Kveberg, B. Rolstad, E.C. Niemi, J.T. Vaage, and J.C. Ryan. 2005. Two structurally related rat Ly49 receptors with opposing functions (Ly49 stimulatory receptor 5 and Ly49 inhibitory receptor 5) recognize nonclassical MHC class Ib-encoded target ligands. *J Immunol* 174:2702-2711.
246. Carlyle, J.R., A. Mesci, B. Ljutic, S. Belanger, L.H. Tai, E. Rousselle, A.D. Troke, M.F. Proteau, and A.P. Makrigiannis. 2006. Molecular and genetic basis for strain-dependent NK1.1 alloreactivity of mouse NK cells. *J Immunol* 176:7511-7524.
247. Hao, L., and M. Nei. 2004. Genomic organization and evolutionary analysis of Ly49 genes encoding the rodent natural killer cell receptors: rapid evolution by repeated gene duplication. *Immunogenetics* 56:343-354.
248. Nylenna, O., C. Naper, J.T. Vaage, P.Y. Woon, D. Gauguier, E. Dissen, J.C. Ryan, and S. Fossum. 2005. The genes and gene organization of the Ly49 region of the rat natural killer cell gene complex. *European journal of immunology* 35:261-272.
249. Wilhelm, B.T., and D.L. Mager. 2004. Rapid expansion of the Ly49 gene cluster in rat. *Genomics* 84:218-221.
250. Sanderson, S., and N. Shastri. 1994. LacZ inducible, antigen/MHC-specific T cell hybrids. *Int Immunol* 6:369-376.
251. Powis, S.J., A.R. Townsend, E.V. Deverson, J. Bastin, G.W. Butcher, and J.C. Howard. 1991. Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter. *Nature* 354:528-531.
252. Kumanovics, A., A. Madan, S. Qin, L. Rowen, L. Hood, and K. Fischer Lindahl. 2002. QUOD ERAT FACIENDUM: sequence analysis of the H2-D and H2-Q regions of 129/SvJ mice. *Immunogenetics* 54:479-489.
253. Makrigiannis, A.P., A.T. Pau, A. Saleh, R. Winkler-Pickett, J.R. Ortaldo, and S.K. Anderson. 2001. Class I MHC-binding characteristics of the 129/J Ly49 repertoire. *J Immunol* 166:5034-5043.
254. Su, R.C., S.K. Kung, E.T. Silver, S. Lemieux, K.P. Kane, and R.G. Miller. 1999. Ly-49CB6 NK inhibitory receptor recognizes peptide-receptive H-2Kb. *J Immunol* 163:5319-5330.
255. Fernandez, N.C., E. Treiner, R.E. Vance, A.M. Jamieson, S. Lemieux, and D.H. Raulet. 2005. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* 105:4416-4423.
256. Kim, S., J. Poursine-Laurent, S.M. Truscott, L. Lybarger, Y.J. Song, L. Yang, A.R. French, J.B. Sunwoo, S. Lemieux, T.H. Hansen, and W.M. Yokoyama. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 436:709-713.

257. Tai, L.H., M.L. Goulet, S. Belanger, A.D. Troke, A.G. St-Laurent, A. Mesci, N. Toyama-Sorimachi, J.R. Carlyle, and A.P. Makrigiannis. 2007. Recognition of H-2K(b) by Ly49Q suggests a role for class Ia MHC regulation of plasmacytoid dendritic cell function. *Molecular immunology* 44:2638-2646.
258. Toma-Hirano, M., S. Namiki, Y. Shibata, K. Ishida, H. Arase, S. Miyatake, K. Arai, and Y. Kamogawa-Schifter. 2009. Ly49Q ligand expressed by activated B cells induces plasmacytoid DC maturation. *European journal of immunology* 39:1344-1352.
259. Abb, J., H. Abb, and F. Deinhardt. 1984. Relationship between natural killer (NK) cells and interferon (IFN) alpha-producing cells in human peripheral blood. Studies with a monoclonal antibody with specificity for human natural killer cells. *Immunobiology* 167:359-364.
260. Facchetti, F., C. de Wolf-Peeters, D.Y. Mason, K. Pulford, J.J. van den Oord, and V.J. Desmet. 1988. Plasmacytoid T cells. Immunohistochemical evidence for their monocyte/macrophage origin. *Am J Pathol* 133:15-21.
261. Liu, P., N.A. Jenkins, and N.G. Copeland. 2003. A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res* 13:476-484.
262. Desrosiers, M.P., A. Kielczewska, J.C. Loredo-Osti, S.G. Adam, A.P. Makrigiannis, S. Lemieux, T. Pham, M.B. Lodoen, K. Morgan, L.L. Lanier, and S.M. Vidal. 2005. Epistasis between mouse Klra and major histocompatibility complex class I loci is associated with a new mechanism of natural killer cell-mediated innate resistance to cytomegalovirus infection. *Nature genetics* 37:593-599.
263. Krug, A., A.R. French, W. Barchet, J.A. Fischer, A. Dzionek, J.T. Pingel, M.M. Orihuela, S. Akira, W.M. Yokoyama, and M. Colonna. 2004. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* 21:107-119.
264. Brown, M.G., A.O. Dokun, J.W. Heusel, H.R. Smith, D.L. Beckman, E.A. Blattenberger, C.E. Dubbelde, L.R. Stone, A.A. Scalzo, and W.M. Yokoyama. 2001. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science (New York, N.Y* 292:934-937.
265. Nakamura, M.C., E.C. Niemi, M.J. Fisher, L.D. Shultz, W.E. Seaman, and J.C. Ryan. 1997. Mouse Ly-49A interrupts early signaling events in natural killer cell cytotoxicity and functionally associates with the SHP-1 tyrosine phosphatase. *The Journal of experimental medicine* 185:673-684.
266. Mason, L.H., J. Willette-Brown, A.T. Mason, D. McVicar, and J.R. Ortaldo. 2000. Interaction of Ly-49D+ NK cells with H-2Dd target cells leads to Dap-12 phosphorylation and IFN-gamma secretion. *J Immunol* 164:603-611.
267. Sjolin, H., S.H. Robbins, G. Bessou, A. Hidmark, E. Tomasello, M. Johansson, H. Hall, F. Charifi, G.B. Karlsson Hedestam, C.A. Biron, K. Karre, P. Hoglund, E. Vivier, and M. Dalod. 2006. DAP12 signaling

regulates plasmacytoid dendritic cell homeostasis and down-modulates their function during viral infection. *J Immunol* 177:2908-2916.

268. Hamerman, J.A., J.R. Jarjoura, M.B. Humphrey, M.C. Nakamura, W.E. Seaman, and L.L. Lanier. 2006. Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells (TREM)-2 and DAP12. *J Immunol* 177:2051-2055.
269. Muller, U., U. Steinhoff, L.F. Reis, S. Hemmi, J. Pavlovic, R.M. Zinkernagel, and M. Aguet. 1994. Functional role of type I and type II interferons in antiviral defense. *Science (New York, N.Y* 264:1918-1921.
270. Andersson, K.E., G.S. Williams, D.M. Davis, and P. Hoglund. 2007. Quantifying the reduction in accessibility of the inhibitory NK cell receptor Ly49A caused by binding MHC class I proteins in cis. *European journal of immunology* 37:516-527.
271. Tai, L.H., M.L. Goulet, S. Belanger, N. Toyama-Sorimachi, N. Fodil-Cornu, S.M. Vidal, A.D. Troke, D.W. McVicar, and A.P. Makrigiannis. 2008. Positive regulation of plasmacytoid dendritic cell function via Ly49Q recognition of class I MHC. *The Journal of experimental medicine* 205:3187-3199.
272. Brune, W., H. Hengel, and U.H. Koszinowski. 2001. A mouse model for cytomegalovirus infection. *Curr Protoc Immunol* Chapter 19:Unit 19 17.
273. Ju, X., M. Zenke, D.N. Hart, and G.J. Clark. 2008. CD300a/c regulate type I interferon and TNF-alpha secretion by human plasmacytoid dendritic cells stimulated with TLR7 and TLR9 ligands. *Blood* 112:1184-1194.