Inhibitory NK Receptor Ly49Q Is Expressed on Subsets of Dendritic Cells in a Cellular Maturation- and Cytokine Stimulation-Dependent Manner

Noriko Toyama-Sorimachi, Yoshiki Omatsu, Atsuko Onoda, Yusuke Tsujimura, Tomonori Iyoda, Akiko Kikuchi-Maki, Hiroyuki Sorimachi, Taeko Dohi, Shinsuke Taki, Kayo Inaba and Hajime Karasuyama

http://www.jimmunol.org/content/174/8/4621

**References**
This article cites 47 articles, 19 of which you can access for free at: http://www.jimmunol.org/content/174/8/4621.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Inhibitory NK Receptor Ly49Q Is Expressed on Subsets of Dendritic Cells in a Cellular Maturation- and Cytokine Stimulation-Dependent Manner\(^1\)

Noriko Toyama-Sorimachi,\(^2\)^\(^*\)† Yoshioki Omatsu,‡ Atsuko Onoda,† Yusuke Tsujimura,\(^*\)^\(^†\) Tomonori Ōyoda,‡ Akiko Kikuchi-Maki,* Hiroyuki Sorimachi,¶ Taeko Dohi,* Shinsuke Taki,§ Kayo Inaba,§ and Hajime Karasuyama\(^†\)

Ly49Q is a member of the Ly49 family that is expressed on Gr-1\(^+\) cells but not on NK and NKT cells. Ly49Q appears to be involved in regulating cytoskeletal architectures through ITIM-mediated signaling. We provide evidence that dendritic cells (DCs) of certain maturation states expressed Ly49Q, and that IFN-\(\alpha\) plays an important role in its regulation. Freshly prepared murine plasmacytoid pre-DCs as well as Flt3L-induced plasmacytoid pre-DCs expressed Ly49Q, whereas freshly prepared myeloid DCs did not. However, GM-CSF-induced myeloid DCs showed low levels of Ly49Q expression, and this was significantly enhanced by plasmacytoid pre-DCs as well as Flt3L-induced plasmacytoid pre-DCs expressed Ly49Q, whereas freshly prepared myeloid DCs were characterized by their ability to secrete high levels of IFN-\(\gamma\), whereas freshly prepared myeloid DCs did not. However, GM-CSF-induced myeloid DCs showed low levels of Ly49Q expression, and this was significantly enhanced by IFN-\(\alpha\). In contrast, other cytokines and ligands for TLRs such as TNF-\(\alpha\), IL-6, LPS, and CpG-ODN had little or no effect on Ly49Q expression. Plasma-activated DCs in all mouse strains examined expressed Ly49Q. Constitutive expression of Ly49Q on myeloid DCs was observed in three restricted mouse strains including 129, NZB, and NZW. As can be seen in other Ly49 family members, Ly49Q expression was affected by MHC class I expression. At the same time, Ly49Q possessed polymorphisms, including at least three alleles. The polymorphic residues lay within the stalk and carbohydrate recognition domain, and two of them, in loop 3 and loop 6 of the carbohydrate recognition domain, are located in the region implicated in the interaction of Ly49A with H-2D\(^d\). Therefore, depending on IFN-\(\alpha\), our results imply that Ly49Q serves a role for the biological functions of certain DC subsets through recognition of MHC class I or related molecules. The Journal of Immunology, 2005, 174: 4621–4629.

The Ly49 family is one of the subfamilies of NK receptors. As with other NK receptor families, the Ly49 family plays a crucial role in self-nonself discrimination by NK cells through its ability to recognize MHC class I or related molecules (1–3). When NK cells recognize MHC class I molecules on target cells, signals transduced by ITIM-bearing inhibitory NK receptors inhibit cytotoxic function and cytokine production (4–8). Ly49Q is an ITIM-bearing inhibitory receptor, which is classified in the Ly49 family because of its high structural similarities and chromosomal location (9). However, Ly49Q has unique features, distinguishing it from other Ly49 family members. We have recently reported that Ly49Q is not expressed on NK and NKT cells, but is predominantly expressed on Gr-1\(^+\) myeloid lineage cells and activated macrophages (9). Expression of Ly49Q on monocytes/macrophages was regulated during their ontogeny and is significantly up-regulated by treatment with IFN-\(\gamma\), which is produced by a wide variety of cells in response to infectious or inflammatory stimuli. Ly49Q has the ability to associate with both Src homology region 2 domain-containing phosphatase 1 and 2 in a tyrosine phosphorylation-dependent manner. Cross-linking of Ly49Q by a specific Ab to Ly49Q triggers rapid cell adhesion and spreading, resulting in the formation of cell polarity in macrophages. Furthermore, Ly49Q can induce tyrosine phosphorylation of various cellular proteins in an ITIM-dependent manner. Therefore, Ly49Q potentially functions as a surface receptor involved in regulation of the cytoskeletal architecture of macrophages, which is essential to macrophage functions such as migration, phagocytosis, production of cytokines, synthesis of bacterialidal materials, and NO.

Dendritic cells (DCs)\(^3\) are the most potent APCs and are essential for the link between innate and acquired immunity (10–13). Murine DCs are characterized by the expression of CD11c and are divided into two major subsets: the CD11c\(^+\)CD11b\(^+\) conventional myeloid DCs and CD11c\(^+\)CD11b\(^-\)B220\(^+\) plasmacytoid pre-DCs, or type I IFN-producing cells (14, 15). Both subsets are distributed throughout various tissues and play different roles in immune responses. Conventional myeloid DCs are able to polarize an adaptive immune response toward a Th1-type response (16). Plasmacytoid pre-DCs are characterized by their ability to secrete high levels of IFN-\(\alpha\), conferring resistance to viruses (14, 15). Both types of DCs express a wide range of receptors, including TLRs and C-type lectins, for the recognition and the subsequent internalization of microbes (17–21). Distinct sets of TLRs and C-type

\(^2\) Department of Gastroenterology, Research Institute, International Medical Center of Japan, Tokyo, Japan; \(^3\) Department of Immune Regulation, Tokyo Medical and Dental University Graduate School, Tokyo, Japan; \(^4\) Department of Animal Development and Physiology, Graduate School of Biostudies, Kyoto University, Kyoto, Japan; \(^5\) Department of Enzymatic Regulation for Cell Functions, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; and \(^6\) Department of Immunology and Infectious Diseases, Shimshu University Graduate School of Medicine, Nagano, Japan

Received for publication August 4, 2004. Accepted for publication January 16, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (15590432, 16043264, and 12051243 to H.K.).

2 Address correspondence and reprint requests to Dr. Noriko Toyama-Sorimachi, Department of Gastroenterology, Research Institute, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. E-mail address: nsorima@ri.imcj.go.jp

3 Abbreviations used in this paper: DC, dendritic cell; CRD, carbohydrate recognition domain; BM, bone marrow; \(\beta_2\)m, \(\beta_2\)-microglobulin.
lectins are expressed on different subsets of DCs depending on their tissue localization and maturational states. Therefore, each differential subset of DCs is specialized to respond to specific stimuli by viruses or microbes.

An increasing number of C-type lectins possessing a carbohydrate recognition domain (CRD) have been identified in both human and mouse DCs (18–21). Their CRD recognizes self or pathogenic structures of glycoproteins. Several C-type lectins, including DC-specific ICAM-3 grabbing nonintegrin, DEC-205, and blood DC Ag 2 are responsible for the endocytosis of a broad array of foreign materials following presentation of Ags to T cells (22–24). However, another C-type lectin known as DC immunoreceptor has a cytoplasmic ITIM, and hence seems to be involved in the negative regulation of DC functions (25). DCs also express structurally different groups of inhibitory receptors, including paired Ig-like receptor B and paired Ig-like type L receptor α/FDFP3, which belong to the Ig-superfamily (26, 27). These inhibitory receptors play crucial roles in tuning the DC functions. For example, DCs were aberrantly activated in PIR-B−/− mice, exacerbating graft-vs-host disease (28). Although ligands of these inhibitory receptors and their exact function in DCs should be further investigated, inhibitory receptors might play important roles in maintaining the homeostasis, initiation, amplification, and termination of DC functions, as observed in other types of immune cells.

In this study, we report that an inhibitory C-type lectin, Ly49Q, was expressed on certain developmental/maturational stages of murine DCs, which was primarily regulated by IFN-α. Our findings illustrate a common recognition system in APCs, such as macrophages and DCs, through the inhibitory receptor Ly49Q.

Materials and Methods

Mice

C57BL/6, BALB/c, C3H, DBA1, and DBA2 mice were purchased fromCLEA Japan. A/J, AKR/N, 129SvJ NZB, NZW, and NZBWF/N mice were purchased from Japan SLC. SJL mice were purchased from Charles River Japan. C57BL/6 β2m−/− mice were purchased from Taconic Farms. JF1 and MSM mice were gifts from Dr. T. Shiroishi (National Institute of Genetics, Shizuoka, Japan). All experiments were performed according to the Guidelines for Animal Use and Experimentation as set out by our institute.

Abs and reagents

Anti-Ly49Q mAb was produced as previously described (9). The following mAbs were purchased from BD Pharmingen: FITC-conjugated anti-CD11b (Mac-1), anti-Gr-1 (Ly6C/G), anti-Ly6C, anti-CD40, anti-CD86, anti-I-Ab, anti-CD4, and anti-CD8e; PE-Cy5.5-conjugated anti-CD11c and streptavidin; biotin-conjugated CD11c; PE-conjugated anti-B220; and allophycocyanin-conjugated streptavidin. FITC, and GL-CSF were purchased from R&D Systems. IFN-α and IFN-γ were purchased from PeproTech. Peptide glycan, LPS, and poly(I:C) were prepared from Sigma-Aldrich. CpG-ODN (TCA TTG GAA AAC GTT CTT CGG GGC G) was phosphorothioate-modified (Hokkaido System Science). Collagenase type III and collagenase (TCA TTG GAA AAC GTT CTT CGG GGC G) was phosphorothioate-modified (Hokkaido System Science). Collagenase type III and collagenase were purchased from Worthington Biochemical and Boehringer Mannheim, respectively.

Cell preparation and culture

In vitro culture of plasmacytoid pre-DCs was performed as described previously (29). Briefly, bone marrow (BM) cells were isolated by flushing femurs and tibiae of euthanized mice with RPMI 1640 medium supplemented with 5% heat-inactivated FCS (Moregate lot no. 49300124; Australia). The BM cells were treated with a Tris-ammonium chloride buffer to lyse RBC and plated at a concentration of 106 cells/ml into a six-well culture dish in culture medium consisting of RPMI 1640 medium supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, 1% (v/v) nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 ng/ml murine Flt3L. Every 4 days of culture, half of the medium was removed and fresh cytokine-supplemented culture medium was added back into the cultures. For preparation of BM-derived myeloid DCs, BM cells were cultured at 2 × 106 cells in 10 ml of the culture medium supplemented with 20 ng/ml GM-CSF.

Twenty-four hours after seeding, 10 ml of fresh medium containing GM-CSF was added, and every 3 days of culture, half of the medium was exchanged. In some experiments, Flt3L- or GM-CSF-induced BM DCs were treated with IFN-α (100 U/ml), IFN-γ (20 ng/ml), TNF-α (50 ng/ml), LPS (1 μg/ml), CpG-ODN D19 (10 μg/ml), or peptide glycan (10 μg/ml) for 18 h, and flow cytometry analysis was performed.

Preparation of DCs from the spleen was performed as previously described (30). Briefly, organs were treated with 1 mg/ml type III collagenase for 30 min at 37°C. After digestion of 10 mg/ml type A collagenase treated organs, they were further incubated at 37°C for 5 min, and dis- persed by gentle pipetting. CD11c+ cells were enriched using AutoMACS cell sorter with anti-CD11c mAb-conjugated microbeads.

Flow cytometry analysis

Immunofluorescence analysis was performed as previously described (9). For staining hemopoietic lineage cells, cells were preincubated with 20% heat-inactivated normal rat serum for 15 min on ice. Incubation of cells with Abs was conducted in the presence of purified 2.4G2 Ab. Cytoplasmic staining was performed in accordance with the company’s protocol using BD Cytofix/Cytoperm kit (BD Biosciences). Stained cells were analyzed with the FACSCalibur system (BD Biosciences).

RT-PCR analysis of Ly49Q transcription and its polymorphisms

Primers for amplifying the full-length Ly49Q sequence were: 5′-CGC GAATTCTAGAAGGACAAGGAGAAGTTACTTTATTCACAA-3′ and 5′-GCC GGTATCTTTAAGGTTGTTGGGACGATCAGCAAG-3′. RNA was prepared from Gr-1+ BM cells and subjected to RT-PCR with these primers. The integrity of mRNAs and successful cDNA synthesis were verified for each sample by monitoring hypoxanthine phosphoribosyltransferase. In some experiments, prepared RNA was treated with RNA-free DNase to eliminate genomic DNA. The PCR products were cloned into a pBS vector using BamHI and EcoR1 recognition sites, and then sequenced using an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Sequences were deposited with GenBank under the following accession numbers: Ly49q1 (AB197779), Ly49q2 (AB19780), Ly49q1 (AB19781), Ly49q1 (AB19782), Ly49q1 (AB19783), Ly49q1 (AB19784), Ly49q1 (AB19785), Ly49q1 (AB19786), Ly49q1 (AB19787), Ly49q1 (AB19788), and Ly49q1 (AB19789).

To classify and confirm nucleotide substitutions in Ly49q1 cDNA prepared from various mouse strains, PCR products were digested with the restriction enzymes indicated and analyzed using gel electrophoresis.

Genomic PCR

Genomic DNA was extracted by incubating mouse tail tissues in an extraction buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 1 mg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween 20, 0.4 mg/ml proteinase K) at 50°C overnight. PCR primers for amplifying Ly49q1 and Ly49q2 were: 5′-GACAGGGACATGACGTTTATCGGCTCT (5′ primer in Fig. 4A) and 5′-CTTGTACACTCTCTTACGAGC-3′ (3′ primer in Fig. 4A). PCR products were cloned into the PBS vector followed by sequence analysis as described above. In some experiments, PCR products were digested with Pst restriction enzyme and analyzed using gel electrophoresis. To determine partial sequences of Ly49q3 (intron 3-exon 4), Pst-digested PCR products obtained from 129 mouse were cloned into the PBS vector followed by sequence analysis. The sequences were deposited with GenBank (Ly49q2, AB193853; Ly49q3, AB193834).

Identification of isoforms and new exons

RT-PCR products corresponding to full-length Ly49Q obtained from JF1, MSM, and 129 × 1/8s mice were cloned into the PBS vector and cDNA sequences of several independent clones which have different sizes of inserts were determined. Existence of sequences corresponding to the newly identified exons was confirmed by searching sequences in the Ensemble genomic sequences of mouse chromosome 6 including KlrA17 (Ensemble gene ID: ENSMUSG000000014543). GenBank accession numbers of isoforms and exons are as follows: Cyt, AB193830; CDR1, AB193831; CDR2, AB193832; and CDR3, AB193834.

Vectors and cDNA transfection

The Ly49Q cDNA modified to add a FLAG tag at the N terminus of Ly49Q was inserted into the eukaryotic expression vector pME10K and then transfected into COS7 cells by electroporation (9). Forty-eight hours after transfection, Ly49Q expression was analyzed using flow cytometry or Western blot analysis.
Results

Expression of Ly49Q on subsets of DCs

Our previous study indicated that a small portion of Gr-1low fraction in BM also expressed Ly49Q (Fig. 1A). To identify these Ly49Q+ Gr-1low cells, further flow cytometry analysis was performed using a panel of Abs against lineage markers. Ly49Q+ Gr-1low cells expressed CD11c as well as B220, suggesting that these cells were plasmacytoid pre-DCs (Fig. 1A, data not shown). As shown in Fig. 1B, most of the B220−CD11c+ plasmacytoid pre-DCs in the BM were specifically stained with anti-Ly49Q mAb. RT-PCR analysis indicated that the mRNA of Ly49Q was also detected in sorted Ly49Q+ B220− cells (data not shown). The expression level of Ly49Q on plasmacytoid pre-DCs exhibited over a 10-fold higher mean fluorescence intensity than that on Gr-1+ cells in BM (9).

Previous studies demonstrated that mouse DCs could be generated in vitro by culturing BM cells with Flt3L or GM-CSF (29, 31). We further confirmed the expression of Ly49Q on plasmacytoid pre-DCs using cultured DCs. Flt3L-induced BM DCs contained several populations, which were divided into groups according to the expression of CD11b and B220. As shown in Fig. 1C, a large portion of CD11c+CD11b+ B220+ plasmacytoid pre-DCs expressed Ly49Q on their surface. CD11c+CD11b+ B220− myeloid DCs also expressed Ly49Q, although the levels from these myeloid DCs were lower than from Flt3L-induced plasmacytoid pre-DCs.

Regulation of Ly49Q expression by IFN-α

We previously reported that Ly49Q expression on monocytes/macrophages is regulated during their differentiation and activation (9). To examine whether the Ly49Q expression is regulated during the DC differentiation/activation process, Flt3L-induced plasmacytoid pre-DCs and GM-CSF-induced myeloid DCs were treated with a panel of cytokines or ligands for TLRs and examined for their expression of Ly49Q. When cells were treated with IFN-α, we observed significant enhancement of Ly49Q expression, as well as MHC class II expression, on CD11c+CD11b+ myeloid DCs (Fig. 2A, right panels, and data not shown). RT-PCR analysis for Ly49Q mRNA by RT-PCR confirmed higher transcription of the Ly49q gene in IFN-α-treated myeloid DCs than in untreated myeloid DCs (Fig. 2, B and C). As for plasmacytoid pre-DCs, Ly49Q expression was slightly enhanced in the presence of IFN-α, IFN-γ, or LPS (Fig. 2A, left and middle panels). In both myeloid and plasmacytoid pre-DCs, IFN-γ was less effective than IFN-α in enhancing the expression of Ly49Q. The enhanced expression of Ly49Q was not observed when both types of DCs prepared from IFNAR−/− mice were treated with IFN-α, even though IFN-γ induced a slight increase of Ly49Q expression on these cells (Fig. 2A and data not shown). Notably, other cytokines and a panel of ligands of TLRs, including TNF-α, Cpg, poly(I:C), peptide glycan, and anti-CD40 mAb had little effect on the expression of Ly49Q, even though these stimuli induced an increased expression of MHC class II (Fig. 2A and data not shown).

We further analyzed Ly49Q expression levels on plasmacytoid pre-DCs in IFNAR−/− mice. However, Ly49Q expression pattern differences on plasmacytoid pre-DCs between IFNAR−/− and wild-type mice was not observed in both the BM and spleen (Fig. 2D).

Expression of Ly49Q in various mouse strains

Previous studies have shown that some members of the Ly49 family are not expressed in some mouse strains, as genes encoding for certain Ly49 were deleted during their diversification (32, 33). Therefore, we investigated the expression of Ly49Q in various mouse strains. Ly49Q was expressed in splenic B220+CD11c+ plasmacytoid pre-DCs prepared from 10 strains of laboratory mice: SJL (H-2b), A/J (H-2b), AKR (H-2k), BALB/c (H-2d), C3H (H-2k), DBA1 (H-2d), DBA2 (H-2b), NZB (H-2b), NZW (H-2b), and 129×1/SvJ (H-2b). Plasmacytoid pre-DCs in wild mice, such as JF1 and MSM, also expressed Ly49Q (data not shown). Correlations between the expression levels and MHC class I haplotypes were not observed, although the expression levels slightly

FIGURE 1. Ly49Q expression on plasmacytoid pre-DCs. A. Three-color flow cytometric analysis of BM cells. BM cells were stained with FITC-conjugated anti-Gr-1 and PE-conjugated anti-CD11c mAbs in combination with biotin-conjugated anti-Ly49Q or control rat IgG2a Abs. CD11c expression on Ly49Q+ Gr-1− cells was analyzed. A thick line in the histogram indicates staining by anti-CD11c. A thin line indicates staining by control Ab, and a dashed line indicates a pattern in the absence of Ab. B. Three-color flow cytometric analysis of BM cells. BM cells were stained with FITC-conjugated anti-CD11c and PE-conjugated anti-B220 mAbs in combination with biotin-conjugated anti-Ly49Q (thick line) or biotin-conjugated rat IgG2a (thin line) revealed by allophycocyanin-conjugated streptavidin. A dashed line indicates the staining pattern in the absence of first Ab. C. Ly49Q expression on BM-derived DCs with Flt3L or GM-CSF. BM-derived DCs were prepared by culturing BM in the presence of Flt3L or GM-CSF as described in Materials and Methods. CD11b+ and CD11b− DCs were purified from Flt3L-induced BM cells using an AutoMACS cell sorter, and then stained with FITC-conjugated anti-CD11c and PE-conjugated anti-B220 mAbs in combination with biotin-conjugated anti-Ly49Q (shaded histograms) or with biotin-conjugated rat IgG2a (open histograms) revealed by allophycocyanin-conjugated streptavidin. GM-CSF-induced DCs were stained with FITC-conjugated anti-CD11c and PE-conjugated anti-CD11b mAbs in combination with biotin-conjugated anti-Ly49Q (shaded histogram) or biotin-conjugated rat IgG2a (open histogram) revealed by allophycocyanin-conjugated streptavidin.
differed by strains (Fig. 3). Interestingly, however, no detectable level of Ly49Q expression on B220+/H11002 CD11c+/H11001 conventional myeloid DCs was observed in most mouse strains, although significant expression of Ly49Q was detected on GM-CSF-induced BM-derived myeloid DCs as described above (Fig. 3B). Exceptionally, in three strains, NZB, NZW, and 129/H110031/SvJ, all myeloid DCs expressed significant yet low levels of Ly49Q relative to plasmacytoid pre-DCs.

Makrigiannis et al. (32) reported that the 129/H110031/SvJ strain has the Ly49q2 gene with a high sequence similarity with Ly49q1. We further examined the possibility that these three strains have the Ly49q2 gene or other related genes, and that myeloid DCs in these strains express them on their surface. Although Ly49q1 and Ly49q2 are highly homologous, these two genes can be discriminated using the PstI restriction enzyme (Fig. 4A). Genomic PCR analysis and subsequent PstI treatments indicated that NZB and NZW mice did not have the Ly49q2 gene in the NK complex (Fig. 4B). Only 129 × 1/SvJ and SJL/J strains have the Ly49q2 gene. In these two mouse strains, we also identified the Ly49q3 gene, which has a high sequence similarity to Ly49q1 and Ly49q2. Because NZB and NZW strains do not have Ly49q2 and Ly49q3 genes, it was strongly suggested that Ly49Q1 proteins were expressed on myeloid DCs in these two strains.

Effect of MHC class I on Ly49Q expression
It has been demonstrated that Ly49 expression levels in NK cells are influenced by MHC class I expression (34–36). To examine whether this is also true for Ly49Q, Ly49Q expression levels on plasmacytoid pre-DCs were compared between C57BL/6 and

FIGURE 2. Enhancement of Ly49Q expression by IFN-α or IFN-γ. A, Flt3L- or GM-CSF-induced BM DCs prepared from wild-type C57BL/6 or IFNαR−/− mice were treated for 18 h with various cytokines or the TLR ligands indicated. Because cells obtained from the cultures contain various subsets, CD11c+CD11b− plasmacytoid pre-DCs in Flt3L-induced DCs and CD11c+CD11b− myeloid DCs in GM-CSF-induced DCs were analyzed for the expression of Ly49Q or MHC class II. Cells were stained with FITC-conjugated CD11c and PE-conjugated anti-CD11b in combination with biotin-conjugated anti-Ly49Q (shaded histograms) or biotin-conjugated rat IgG2a mAbs (solid line in the (-) histogram) revealed by streptavidin-PC5. For MHC class II staining, cells were stained with PE-conjugated anti-CD11c and PC5-conjugated anti-CD11b mAbs in combination with FITC-conjugated anti-MHC class II (shaded histograms) or control mAbs (open histograms). All shaded histograms except for (-) histograms are overlaid with open histograms; these indicate patterns of untreated DCs stained with anti-Ly49Q mAb (solid lines). B, GM-CSF-induced BM DCs were treated with IFN-α or IFN-γ for 18 h and stained with anti-Ly49Q or anti-MHC class II mAbs as described above. Shaded histograms indicate staining patterns with anti-Ly49Q or anti-MHC class II mAbs. Open histograms indicate staining patterns with isotype-matched control mAbs. C, mRNA was prepared from IFN-α-stimulated myeloid DCs, and then RT-PCR was performed using primer sets specific for Ly49Q as described previously (9). Templates were used in serial 5-fold dilutions. D, DCs were prepared from BM and spleens of IFNαR−/− and control mice, and Ly49Q expression was examined by flow cytometry analysis.

FIGURE 3. Ly49Q expression on plasmacytoid pre-DCs and myeloid DCs in various mouse strains. DCs in a steady-state condition were prepared from spleens of various mouse strains by collagenase treatment, and stained with FITC-conjugated anti-CD11c and allophycocyanin-conjugated anti-B220 mAbs in combination with biotin-conjugated anti-Ly49Q (shaded histograms) or control rat IgG2a (open histograms). Histograms show the level of Ly49Q expression on CD11c+ B220+ plasmacytoid pre-DCs (A) or on CD11c+ B220+ myeloid DCs (B).
Polymorphisms and isoforms of Ly49Q

To obtain clues for a possible ligand for Ly49Q, a polymorphic variation of Ly49Q was examined. Ly49Q cDNA was additionally cloned from 10 laboratory and 2 wild mouse strains, and we determined their nucleotide sequences. To exclude the possibility that nucleotide substitutions in obtained sequences were PCR artifacts, multiple independent clones were used for determining the sequences. Furthermore, restriction enzymes that could discriminate nucleotide substitutions in different alleles were chosen by comparing sequences, and allelic variations in mouse strains were confirmed by digesting the PCR products corresponding to Ly49Q with restriction enzymes (Fig. 6). We identified at least three alleles, tentatively termed Ly49q1a, q1b, and q1c, possessing nucleotide variations at 10 residues and amino acids variations at 7 residues (Fig. 7A). Four of the seven amino acid variations were identified within stalk regions, and three within the CRD region. The cytoplasmic regions, including ITIMs, were completely conserved. No correlations between MHC haplotypes and Ly49Q allele types were observed (Fig. 7B). Based on previous studies showing crystal structures of Ly49A and Ly49C, polymorphisms at position 229 and at position 253, respectively, resided in loops 3 and 6, which are important for recognition of MHC class I and β2m by Ly49A (Fig. 7C) (37–39).

We further identified three additional exons in the Ly49q1 gene and four additional splice variants of Ly49Q1 in mouse strains 129, JF1, and MSM (Fig. 8, A and B). To examine the surface expression of newly identified alleles and isoforms, FLAG-tagged Ly49Q1 cDNA was introduced into COS7 cells, their expression was tested using anti-Ly49Q and anti-FLAG mAbs (Fig. 8C). All three alleles of Ly49q1 were expressed on the surface and recognized by anti-Ly49Q mAb as well as by anti-FLAG mAb, although the efficiency of Ly49Q1c expression was lower than that of other two alleles. Expression of the four isoforms was not detected using anti-Ly49Q mAb and anti-FLAG mAb. In biochemical analysis of these isoforms, degradation products derived from FLAG-tagged proteins was detectable, suggesting that these isoforms are not stably expressed in cells (data not shown).

Discussion

We have demonstrated that an ITIM-bearing inhibitory receptor, Ly49Q, was expressed on DC subsets. Freshly isolated plasmacytoid pre-DCs expressed Ly49Q on their surface. In contrast, in most mouse strains, freshly prepared myeloid DCs did not express Ly49Q. However, GM-CSF-induced myeloid DCs prepared from BM expressed low but significant levels of Ly49Q. Of particular interest, expression of Ly49Q was greatly enhanced when DCs were treated with IFN-α or IFN-γ. A panel of ligands for TLRs

FIGURE 5. Comparison of Ly49Q expression levels between β2m−/− and control mice. DCs were prepared from the spleen and BM as described in Materials and Methods. CD19−/B220− cells were gated and Ly49Q expression was examined. Numeric data in histograms indicate the mean fluorescence intensity of histograms representing staining patterns using control (left side) or anti-Ly49Q (right side) mAbs.
had minimal effect on Ly49Q expression in our assay conditions. Therefore, DCs express Ly49Q depending on their maturational/activation states and on their cytokine environments. This also implies a potential role of Ly49Q in immune responses under certain circumstances such as virus infection.

Ly49Q expression seems to be regulated through the differentiation and activation process of DC subsets, as has been observed in monocytes/macrophages (9). Flt3L-induced BM-derived plasmacytoid pre-DCs expressed lower levels of Ly49Q than freshly prepared plasmacytoid pre-DCs from the BM or spleen. Based on their lower levels of surface expression of CD80, CD86, and MHC class II and of their lower production of IFN-γ, Flt3L-cultured BM cells are considered less mature than those in peripheral lymphoid organs (40). Therefore, expression of Ly49Q on plasmacytoid pre-DCs appears to be enhanced as they mature. Compared with plasmacytoid pre-DCs, myeloid DCs showed a different pattern of Ly49Q expression. Because detectable levels of Ly49Q were observed on GM-CSF-cultured BM-DCs but not on freshly prepared myeloid DCs from spleens, we speculate that the surface expression of Ly49Q decreases during differentiation in myeloid DCs. Treatment of GM-CSF-cultured BM-DCs with IFN-α induced a substantial increase in Ly49Q expression, suggesting that myeloid DCs re-express Ly49Q in the presence of IFN-α. In addition, myeloid DCs in some mouse strains expressed Ly49Q on their surface. Taken together, it is likely that Ly49Q plays a role not only in plasmacytoid pre-DCs, but also in myeloid DCs in some circumstances. Expression of other inhibitory receptors such as PIR-A/B and SIRP on GM-CSF- and Flt3L-cultured BM-DCs, as well as Ly49A, Ly49C, and CD94 on NK cells, was not affected by the treatment with IFN-α (data not shown). The selective enhancement of Ly49Q expression by IFN-α or IFN-γ may imply potential roles for Ly49Q in immune responses elicited by virus infections. Further investigations are necessary to clarify this.

**FIGURE 7.** Alignment of Ly49q1 sequences from various mouse strains. A. Amino acid sequences of the Ly49q1 gene from various mouse strains were determined. Amino acids possessing allelic variations are shown in boxes. Three alleles of Ly49q1 were identified. B. Types of Ly49q1 alleles in various mouse strains are shown. C. Positions of polymorphic residues of Ly49Q. Asterisks denote contact sites of Ly49A with H-2Dd (37). Lys224 and Val248 of Ly49A, respectively correspond to Thr/Lys229 and Val/Phe253 polymorphic residues in Ly49Q.
Temporal expression of Ly49Q during differentiation/ maturation processes of myeloid DCs seems to parallel that of monocytes/macrophages (9). Gr-1+ immature myeloid cells, including immature monocytes, expressed high levels of Ly49Q in the BM. Expression of Ly49Q seemed to disappear in circulating monocytes in peripheral blood, and then appeared after activation. Similarity of Ly49Q expression patterns suggests similar functions of Ly49Q in DCs and in macrophages. Our previous study demonstrated that Ly49Q itself introduces signals from extracellular to intracellular to rearrange the cytoskeleton, resulting in rapid spreading and formation of pseudopods in IFN-γ-activated macrophages (9). We observed a similar effect of anti-Ly49Q mAb on the morphology of IFN-α-stimulated plasmacytoid pre-DCs (data not shown). We also observed that anti-Ly49Q mAb used in this study had no effect on the production of IFN-α and other cytokines by plasmacytoid pre-DCs (data not shown). Although further analysis is required to clarify the function of Ly49Q in macrophages and DC subsets, our findings suggest a unique common regulatory mechanism in professional APCs, such as macrophages and DCs, for regulating cytoskeleton and cell adhesiveness through an inhibitory receptor.

Compared with other C-type lectins expressed on DCs, such as DEC-205 and DC-SIGN, Ly49Q seems to play distinct roles. DEC-205, Langerin (CD207), Lox-1, and mouse macrophage galactose-type C-type lectin recognize carbohydrate profiles on microorganisms, and are involved in internalization, processing and presentation of foreign Ags (41–45). Although Ly49Q does not possess a typical leucine-based internalization motif in its cytoplasmic region, the YxxL sequence in its cytoplasmic ITIM could be related with a tyrosine-based internalization motif Yxxφ, where φ is any bulky hydrophobic residue (46). However, involvement of Ly49Q in Ag uptake may be less possible. Incubation of murine macrophage cell lines with anti-Ly49Q mAbs together with anti-DEC-205 mAbs did not induce an internalization of this molecule from the surface of macrophage cell lines (A. Kikuchi-Maki and N. Toyama-Sorimachi, unpublished observation). Therefore, Ly49Q appears not to be involved in Ag uptake or internalization. In addition, Ly49Q, like other Ly49 family members, does not contain either the essential motif necessary for recognition of mannose- or galactose-containing carbohydrates, or amino acid residues involved in calcium binding, which is conserved in other calcium-dependent lectins. Therefore, it is reasonable to assume that Ly49Q is not associated with recognition and internalization of microbes via carbohydrate structures.

We now know that inhibitory Ly49 family members expressed on NK and NKT cells recognize MHC class I molecules and are responsible for self-nonself discriminations (1–3). Based on the high structural similarity between Ly49Q and other conventional Ly49 family members expressed on NK and T cell subsets, we hypothesize that Ly49Q also recognizes MHC class I or its related molecules. Based on this point of view, it is of particular interest to determine whether Ly49Q possesses allelic polymorphisms. Sequence analysis of Ly49Q cDNA isolated from various mouse strains indicated that Ly49Q is a polymorphic receptor, similar to other Ly49 family members, suggesting that the ligand for Ly49Q may be a polymorphic molecule. Structural analysis from crystallography indicated that loops 1, 5, and β-sheet 4 are important in the recognition of MHC class I by both Ly49A and Ly49C (37, 38). In addition, in the case of Ly49A, loops 3 and 6 are important for the recognition of MHC class I and the associated βm (37). In the case of Ly49A, valine at position 253 is especially important in interaction with βm. Importantly, amino acid substitutions between polymorphic alleles at position 239 and 253 reside in loops 3 and 6, respectively. Therefore, we speculate that the polymorphic variations in Ly49Q may influence its ligand recognition.

Previous studies have demonstrated that expression levels of other Ly49 molecules on NK cells were apparently regulated by corresponding MHC class I molecules (receptor calibration model) (34–36). Our comparison of expression levels of Ly49Q in various mouse strains showed that there was no correlation between the expression levels of Ly49Q and MHC class I haplotypes. However, expression levels of Ly49Q were affected by the lack of βm, suggesting that a ligand for Ly49Q is a βm-associated molecule. Interestingly, the effect of βm on Ly49Q expression was observed only in the spleen, implying that Ly49Q expression was conditioned during the plasmacytoid pre-DC maturation process. Although a ligand for Ly49Q is yet to be unidentified, it is likely that both the ligand and the cytokine environment exert an influence on the Ly49Q expression level because IFNs greatly enhanced the Ly49Q expression levels.

Ly49Q expression was widely observed in plasmacytoid pre-DCs in all mouse strains examined, suggesting the importance of this molecule in these cells. Of interest, freshly prepared myeloid DCs in mouse strains 129, NZB, and NZW expressed considerably higher levels of Ly49Q in a steady state. Our analysis indicated that Ly49Q1 has several isoforms and alleles. In addition, Ly49q2 and q3 genes exist in some mouse strains. Therefore, exceptional staining of myeloid DCs by anti-Ly49Q mAb may be due to the
expression of Ly49Q1-related molecules including Ly49Q2, Q3, and isoforms of Q1. However, genomic and RT-PCR analysis demonstrated that Ly49Q2 and Q3 were not expressed in DCs in mouse strains 129, NZB, and NZW. Furthermore, in our reconstitution experiments, Ly49Q1 isoforms appeared not to be expressed. Therefore, rather than being due to cross-reactivity of anti-Ly49 mAb to Ly49Q1-related molecules on myeloid DCs, it seems to be more likely that Ly49Q1 expression is elicited on myeloid DCs because of cytokine environment differences present in these three mouse strains. Previous observation showing that 129 mice produce a higher amount of IFN-α compared with other laboratory mouse strains may support this notion (47).

In conclusion, Ly49Q is an inhibitory receptor expressed on DC subsets. Expression is regulated during DC differentiation/maturity. IFN-α is the most potent inducer of Ly49Q, suggesting important roles of this molecule in combating viral infection. The possible roles of Ly49Q in regulation of the cytokines are particularly interesting because reorganization of cytokine network is an important event in DC functions including migration, Ag presentation, and cytokine production. Further investigation to gain insights into the physiological roles of Ly49Q will help elucidate cytokine regulatory function of DCs as well as a novel recognition mechanism for DCs through a MHC class I receptor.

Acknowledgements
We thank Dr. A. Makrigiannis for helpful discussion, Dr. T. Shiroishi for giving us JF1 and MSM mice, and M. Hayashi, M. Nakasuji, and K. Hirai for technical supports.

Disclosures
The authors have no financial conflict of interest.

References


