Ly49Q, a member of the Ly49 family that is selectively expressed on myeloid lineage cells and involved in regulation of cytoskeletal architecture

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Here, we identified and characterized a Ly49 family member, designated as Ly49Q. The *Ly49q* gene encodes a 273-aa protein with an immunoreceptor tyrosine-based inhibitory motif (ITIM) at the N terminus of its cytoplasmic domain. We show that the ITIM of Ly49Q can recruit SHP-2 and SHP-1 in a tyrosine phosphorylation-dependent manner. In contrast to other known members of the Ly49 family, Ly49Q was found not to be expressed on NK1.1$^+$ cells, but instead was detectable on virtually all Gr-1$^+$ cells, such as myeloid precursors in bone marrow. Monocytes/macrophages also expressed low levels of Ly49Q, and the expression was enhanced by the treatment of cells with IFN-γ. Treatment of activated macrophages with anti-Ly49Q mAb induced rapid formation of polarized actin structures, showing filopodia-like structure on one side and lamellipodial-like structure on the other side. A panel of proteins became tyrosine-phosphorylated in myeloid cells when treated with the mAb. Induction of the phosphorylation depends on the ITIM of Ly49Q. Thus, Ly49Q has unique features different from other known Ly49 family members and appears to be involved in regulation of cytoskeletal architecture of macrophages through ITIM-mediated signaling.

The immune system should be tightly regulated to avoid overresponses that result in serious immune diseases such as autoimmunity and inflammation. It is now well established that inhibitory receptors play critical roles for fine-tuning of immune responses (1–3). A number of inhibitory receptors have been identified on various types of cells. In particular, inhibitory receptors on natural killer (NK) cells have been well characterized. It has been postulated that recognition of MHC class I, or related molecules, on target cells by inhibitory NK receptors allows NK cells to prevent self-killing but destroy inappropriate cells possessing decreased levels of MHC class I (4–6). In mice, two types of inhibitory receptors on NK cells have been identified that belong to the Ly49 family and CD94/NKG2 family (7–9).

Ly49 family members are expressed on subsets of NK and NKT cells as disulfide-linked dimers (7–9). The Ly49 family belongs to a group of type II integral membrane protein that contains external domains homologous to the superfamily of Ca^{2+}-dependent lectins (10). In the cytoplasmic domains, they contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) that engages the intracellular tyrosine phosphatases, SHP-1 or SHP-2 (11–13). However, not all Ly49 molecules contain an ITIM. Some Ly49 family members do not display ITIMs and instead activate NK cells. Ly49 genes are highly polymorphic and located within the NK complex, a stretch of 2 Mb on mouse chromosome 6 as a complex multigene family (14–16). This NK complex region is conserved among species, having been identified on syntenic regions of rat and human chromosomes (17). The complexity and polymorphism of the Ly49 family have not been fully analyzed, and additional genes having similar sequences to those of already characterized Ly49 cDNA clones may exist.

Here, we describe a Ly49 molecule, designated as Ly49Q, cloned from fetal liver mononuclear cells (MNCs). Flow cytometric and functional analyses with anti-Ly49Q mAbs indicated that Ly49Q is distinct from other members of the Ly49 family. Our results suggest an additional function of Ly49 family outside of NK cells.

Materials and Methods

Mice. C57BL/6 mice were purchased from CLEA Japan (Tokyo). Dark Agouti rats were purchased from Charles River Japan (Kanagawa, Japan). All experiments were performed according to Guidelines for Animal Use and Experimentation as set out by Tokyo Medical and Dental University.

Cell Preparation and Cell Culture. Peritoneal exhausting cells (PECs) were collected with cold PBS containing 0.05% EDTA at 4 days after i.p. injection of 1 ml of 3% thioglycorate medium. Fetal liver MNCs were prepared by the lympholyte density method (18). Cells were cultured in complete RPMI medium 1640 as described (19).

Isolation and Sequence Analysis of Ly49Q cDNA Clone. A pair of oligonucleotide primers for RT-PCR was synthesized that corresponded to the nucleotide sequences of the Ly49C gene derived from C57BL/6 mice: 5′-GATGAGTGAGCCAGAGGTCACTTAC-3′ and 5′-TTAATCAGGGAATTTATCCAGTTTCTTCCCAC-3′. RNA was prepared from C57BL/6 fetal liver MNCs on gestation day 16 and subjected to RT-PCR with the primers. The PCR products were cloned into pGEM-T vector (Promega). A cDNA library prepared from C57BL/6 fetal liver MNCs of gestation day 16 by using ZAP II (Stratagene) was screened by using the PCR-amplified fragment as a probe. 5′ RACE-PCR was further performed to obtain a full-length cDNA of Ly49 (designated as Ly49Q).

Vectors and cDNA Transfection. The Ly49Q cDNA with the FLAG tag at the N terminus was inserted into an eukaryotic expression vector pME18S and transfected into COS7 cells by electroporation (20). Expression of Ly49Q was analyzed by flow cytometry or Western blot analysis 48 h after transfection. In retroviral

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Abbreviations: NK, natural killer; MNC, mononuclear cell; ITIM, immunoreceptor tyrosine-based inhibitory motif; PE, phycoerythrine; PEC, peritoneal exhausting cell; TGC, thioglycollate.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AB033769).

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expression system, pMx-IRES-GFP vector was used for WEHI3 cells (21).

Establishment of mAb Against Ly49Q. Dark Agouti rats were immunized at weekly intervals by footpad injection of Ly49Q-transfected COS7 cells (1–2 × 10^7 cells per animal). Immunizations and cell fusion were performed as described (19). Hybridomas were screened by their ability to specifically stain Ly49Q-transfected COS7 cells.

Antibodies. The following mAbs were purchased from BD Pharmingen: FITC-conjugated anti-Mac-1 and anti-Gr-1; phycoerythrine (PE)-conjugated anti-NK1.1; streptavidin-conjugated allophycocyanin and PE; and control rat IgG2a and IgG2b. Agarose beads conjugated with anti-FLAG antibody M2, antiphosphotyrosine antibody 4G10, biotin-conjugated M2, and 4G10 were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-mouse CD44 IM7 has been described (22). Anti-Gr-1-coated microbeads for magnetic cell sorting were purchased from Miltenyi Biotec (Gladbach, Germany). Anti-SHP-1 and -2 antibodies were purchased from Santa Cruz Biotechnology.

Flow Cytometric Analysis. Immunofluorescence analysis was performed as described (19). Cytoplasmic staining was performed according to the manufacturer's instructions for a Cytofix/Cytoperm Kit (BD Biosciences, San Diego). Stained cells were analyzed with FACSCalibur (Becton Dickinson). Cell sorting was performed with a FACSVantage (Becton Dickinson). Magnetic cell sorting was performed with an AutoMACS (Miltenyi Biotec).

PCR Analysis. Ly49Q-specific primers used were 5′-GAGAAGCTGGCCACAAAGAGTGCTCTATC-3′ and 5′-CAGCATTTCTTCCTTTGCGTTGATGTCAT-3′. Ly49A-specific primers were 5′-AAGTCTATGGAGTGTGATC-3′ and 5′-AATACTTGATCACAGTTACC-3′. NK1.1-specific primers were 5′-GCCCACAAGACTGGCTTTTACACCGAG-3′ and 5′-GTCTGAAGCACAGCTCTCAGGAGTCAC-3′. The integrity of mRNAs and successful cDNA synthesis were verified for each sample by monitoring hypoxanthine phosphoribosyltransferase (23). Some of the PCR products were cloned into pBS vector followed by sequence analysis by using an ABI PRISM 310 Genetic Analyzer (Perkin–Elmer Applied Biosystems).

Cell Surface Labeling and Immunoprecipitation. Cells were surface-labeled with biotin (Pierce) and lysed in 1% Nonidet P-40 lysis buffer (24). Immunoprecipitation and Western blot analysis were performed as described (24). The biotinylated proteins were detected with horseradish peroxidase-conjugated streptavidin (Amersham Pharmacia) and visualized by ECL-system (Amersham Pharmacia). In the analysis of phosphotyrosine, SuperSignal West Dura Extended Duration Substrate was used for detection (Pierce).

Analysis of Tyrosine-Phosphorylated Proteins. COS7 transfectants were suspended in cold 5% FCS/PBS at 2 × 10^6 cells per 75 μl. After addition of 25 μl of 4 mM pervanadate, the cells were incubated at 37°C for 5 min, followed by the addition of 1 ml of 1% Nonidet P-40 lysis buffer containing 100 mM NaF and 1 mM Na_3VO_4. The cell lysates were subjected to immunoprecipitation and immunoblotting. In the case of tyrosine phosphorylation in WEHI3 transfectants, cells were treated with 100 units/ml IFN-γ for 16 h before the assay and placed into wells of Falcon six-well plates precoated with anti-Ly49Q or control antibodies (20 μg/ml). After incubation at 37°C for indicated periods, cell lysates were prepared.

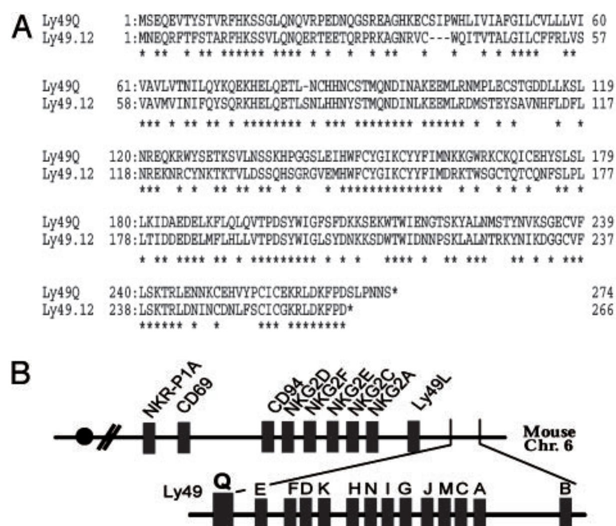

Fig. 1. Amino acid sequence of Ly49Q and Ly49 gene map. (*A*) The predicted amino acid sequence of the Ly49Q was aligned with that of rat Ly49.12, which has the most closely matching amino acid sequence to Ly49Q. Conserved residues are marked by *. (*B*) Schematic map of Ly49 genes of C57BL/6. The relative location of Ly49 family members are shown, but distances between them are approximate. The B6 gene map is reproduced from available GenBank bacterial artificial chromosome sequence data and previous reports (15, 16). The Ly49Q gene is located at the centromeric end of the Ly49 cluster.

Cell Adhesion Assay. WEHI3 transfectants or RAW264 cells (5 × 10^5 cells per 1.5 ml per well in a six-well plate) were added into antibody-coated wells, and then incubated at 37°C for indicated periods. The extent of cell spreading was evaluated by measuring a major/minor axis ratio.

Results

cDNA Cloning of a Ly49 Gene, *Ly49Q*. To identify NK receptors expressed on immature NK cells, PCR-based cDNA cloning of Ly49 was performed by using RNA from fetal liver MNCs. Among 10 randomly chosen PCR products, 6 were identical in their sequence but were distinct from sequences of known Ly49 family members. The nucleotide sequence of the full-length cDNA corresponding to the PCR product shows an ORF of 273 aa (Fig. 1) with high similarity to other members of the Ly49 family [61% identity to rat Ly49.9 (25), 58% to rat Ly49.29 (25), 55% to mouse Ly49C (26)]. The predicted protein had the characteristics of a type II integral membrane protein and five sites for potential N-glycosylation in the extracellular region. It also possesses a typical ITIM in the cytoplasmic domain, from positions 6 to 11 (VxYxxV) in the sequence. These results indicated that the cDNA encoded a Ly49 family member that had not been reported previously. Therefore, we designated this molecule Ly49Q and deposited the sequence corresponding to the full-length Ly49Q cDNA in GenBank. Subsequent studies identified the gene locus of Ly49Q in both C57BL/6 and 129 strains (15, 16) and showed that the Ly49Q gene is located at the centromeric end of Ly49 cluster (chromosome 6) (Fig. 1*B*).

Biochemical Characterization of Ly49Q Protein. COS7 cells were transfected with either FLAG-tagged Ly49Q cDNA or mock plasmid. Anti-FLAG antibody stained only Ly49Q transfectants (Fig. 2*A*) and immunoprecipitated cell surface Ly49Q as 45- to 60-kDa molecules when analyzed under reduced conditions (Fig. 2*B*). Under nonreducing conditions, FLAG-tagged Ly49Q showed an apparent molecular mass of 130 and 220 kDa, indicating that Ly49Q is expressed as dimer or oligomer on the cell surface as in case of other Ly49 family members (27, 28).

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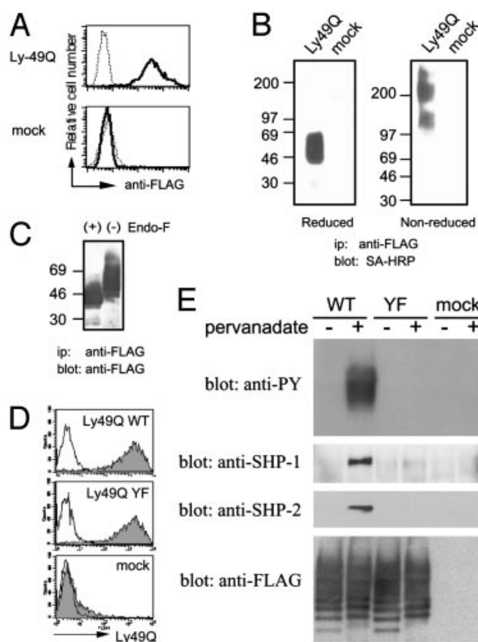

Fig. 2. Biochemical analysis of Ly49Q protein in COS7 cells. (*A*) Flow cytometry of COS7 cells transfected with the cDNA encoding FLAG-tagged Ly49Q stained with anti-FLAG mAb. (*B*) Gel electrophoresis of proteins from cell lysates of COS7 cells transfected with FLAG-Ly49Q cDNA surface-labeled with biotin and immunoprecipitation with anti-FLAG mAb. The gels were blotted, reacted with horseradish peroxidase-conjugated streptavidin, and stained with the peroxidase reaction. (*C*) Biotin-labeled immunoprecipitates with anti-FLAG mAb as in *B* were treated with or without N-glycanase. (*D*) Flow cytometry of COS7 cells transfected with Ly49Q WT cDNA, Ly49Q YF mutant cDNA, or empty vector. Cells were stained with anti-FLAG mAb. (*E*) Cell lysates prepared from COS7 transfectants were subjected to immunoprecipitation with anti-FLAG-mAb. The precipitates were blotted with the Abs indicated.

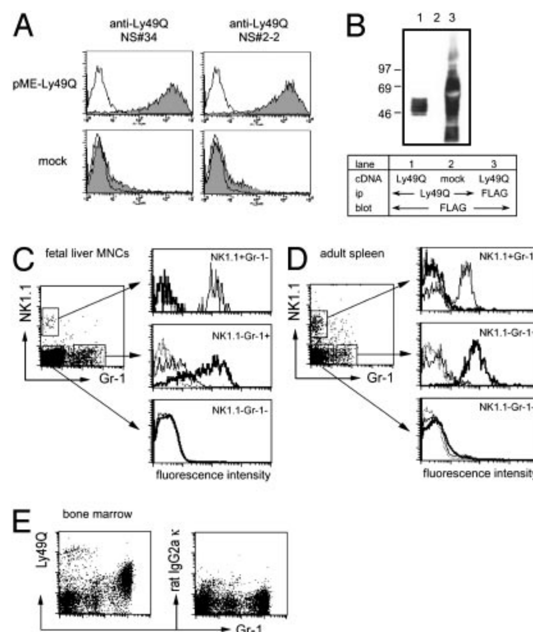

Fig. 3. Expression of Ly49Q on Gr-1$^+$ cells but not on NK cells. (*A*) Flow cytometric analysis of COS7 cells transfected with Ly49Q cDNA or mock cDNA using biotin-conjugated anti-Ly49Q mAb 34 (rat isotype IgG2a,k, shaded histogram, *Left*), or mAb 2-2 (rat isotype IgG2b,k, shaded histogram, *Right*) stained with PE-conjugated streptavidin. Cells for controls were reacted with biotin-conjugated mAbs isotype-matched rat IgG (open histograms). (*B*) Gel electrophoresis analysis of anti-Ly49Q mAb 34 immunoprecipitates of cell lysates of COS7 cells transfected with FLAG-Ly49Q cDNA and surface-labeled with biotin. The precipitated proteins were analyzed by blotting with horseradish peroxidase-conjugated streptavidin. (*C* and *D*) Flow cytometry of cells from fetal liver (*C*) and adult spleen (*D*) stained with FITC-conjugated NK1.1, PE-conjugated anti-Gr-1, and biotin-conjugated anti-Ly49Q mAb 34 (thick lines), anti-2B4 mAb (thin lines), or isotype-matched control rat IgG (dashed lines). Biotin-conjugated antibodies were revealed with allophycocyanin (APC)-conjugated streptavidin. (*E*) Flow cytometry of cells from adult bone marrow stained with PE-conjugated anti-Gr-1 and biotin-conjugated anti-Ly49Q (*Left*) or control biotin-conjugated rat IgG2a,k (*Right*) revealed with APC-conjugated streptavidin.

Ly49Q appeared to be N-glycosylated because the treatment with N-glycosidase F reduced its apparent molecular mass (Fig. 2*C*).

It is well established that some Ly49 family members having an ITIM associate with Src homology 2 domain-containing tyrosine phosphatases such as SHP-1 and SHP-2 (7, 8). To examine whether Ly49Q could engage phosphatases in the cytoplasmic ITIM, COS7 cells were transfected with either FLAG-tagged Ly49Q WT cDNA or Ly49Q YF mutant cDNA in which the tyrosine residue in the ITIM was substituted to phenylalanine. Levels of expression of Ly49Q WT and Ly49Q YF in transfectants were comparable as detected by anti-FLAG mAb (Fig. 2*D*). Treatment of the transfectants with pervanadate induced phosphorylation of the Ly49Q WT protein but not the Ly49Q YF mutant protein, indicating that the tyrosine residue in the ITIM was phosphorylated by activation stimuli (Fig. 2*E*). Both SHP-1 and SHP-2 were coimmunoprecipitated with Ly49Q when COS7 cells expressing the Ly49Q WT were treated with pervanadate, whereas these phosphatases were not coprecipitated from COS7 cells expressing the Ly49Q YF mutant even with pervanadate stimulation (Fig. 2*E*). These results indicate that phosphorylation of the tyrosine residue in the ITIM is necessary for recruitment of SHP-1 and SHP-2 to Ly49Q. Thus, Ly49Q expressed on COS7 cells shows biochemical characteristics similar to other known Ly49 family members (12, 27–29).

Ly49Q Was Expressed on Gr-1-Positive Cells but Not on NK Cells. We established mAbs specific for Ly49Q by immunizing rats with COS7 cells expressing Ly49Q on their surface. As shown in Fig. 3*A*, two mAbs, NS 34 and NS 2-2, stained Ly49Q transfectants but not mock transfectants. Both anti-Ly49Q mAbs precipitated Ly49Q from lysates of Ly49Q transfectants (Fig. 3*B* and data not shown). These results indicate NS 34 and NS 2-2 recognize Ly49Q. Because both anti-Ly49Q mAbs showed similar reactivity to Ly49Q expressed on COS7 cells, NS 34 was used for further characterization of Ly49Q in the present study.

We expected that the anti-Ly49Q mAb NS 34 would stain NK1.1$^+$ cells in fetal liver, because we cloned the Ly49Q cDNA from fetal liver with the aim of identifying novel NK receptors expressed on immature NK cells. However, none of the NK1.1$^+$ cells in fetal liver express molecules recognized by NS 34 (Fig. 3*C*). Surprisingly, essentially all Gr-1$^+$ cells were stained with NS 34, whereas control rat IgG did not stain any Gr-1$^+$ cells. An antibody specific for NK activation receptor 2B4 did not stain Gr-1$^+$ cells even though it stained all NK1.1$^+$ cells. In adult spleen, expression of Ly49Q was also observed in all Gr-1$^+$ cells (Fig. 3*D*). No splenic NK1.1$^+$ cells or NK1.1$^-$Gr-1$^-$ cells expresses molecules recognized by NS 34, indicating that Ly49Q is not expressed on NK cells. This finding also indicates that NS 34 has no cross-reactivity with other Ly49 family members expressed on NK cells. Gr-1$^+$ cells in bone marrow also expressed Ly49Q on their surface (Fig. 3*E*).

Expression of Ly49Q in Gr-1$^+$ cells was further examined by RT-PCR analysis. Gr-1$^+$ and Gr-1$^-$ cells were separated from bone marrow by magnetic cell sorting (Fig. 4*A*). Subsequently

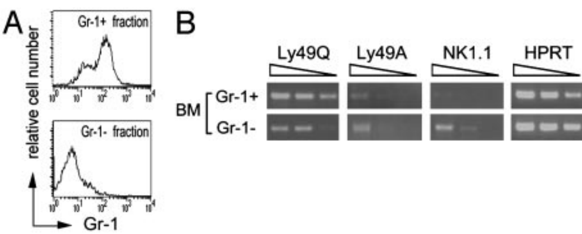

Fig. 4. Expression of Ly49Q mRNA in leukocyte subsets. Gr-1$^+$ and Gr-1$^-$ cells were separated from bone marrow cells by magnetic cell sorting using Gr-1-specific beads. (*A*) Cytometry of separated cells stained with FITC-conjugated anti-Gr-1 mAb. (*B*) RT-PCR analysis with the primers specific for either Ly49Q or Ly49A. RNA was prepared from Gr-1$^{+/-}$ bone marrow (BM) cells. HPRT, hypoxanthine phosphoribosyltransferase.

semiquantitative RT-PCR analysis was performed to detect transcription of the Ly49Q gene (Fig. 4*B*). PCR primers designed for Ly49Q amplified only Ly49Q, whereas primers for Ly49A amplified only Ly49A, but not Ly49B, Ly49C, Ly49D, Ly49E, Ly49F, or Ly49G family members when cloned Ly49 cDNAs were used as templates (data not shown). Transcription of the Ly49Q gene was detected in Gr-1$^+$ cells in bone marrow (Fig. 4*B*). Sequence analysis demonstrated that the PCR products cloned from Gr-1$^+$ cells indeed encoded Ly49Q (data not shown). Taken together with results from flow cytometric analysis, we concluded that Ly49Q is indeed expressed on Gr-1$^+$ cells.

Expression of Ly49Q on Macrophages and Its Enhancement by IFN-γ. Peripheral blood MNCs were divided into at least five populations based on expression patterns of Gr-1 and Mac-1 (30). As shown in Fig. 5*A*, the highest expression of Ly49Q was observed in Gr-1^{high}Mac-1^{high} cells. The expression of Ly49Q was lower in Gr-1^{low}Mac-1^{high} population, and Mac-1$^+$Gr-1$^-$ and Mac-1$^-$Gr-1$^+$ cells expressed very low or no Ly49Q on their surface (Fig. 5*A*). The Mac-1$^+$Gr-1$^-$ population seemed to be mature

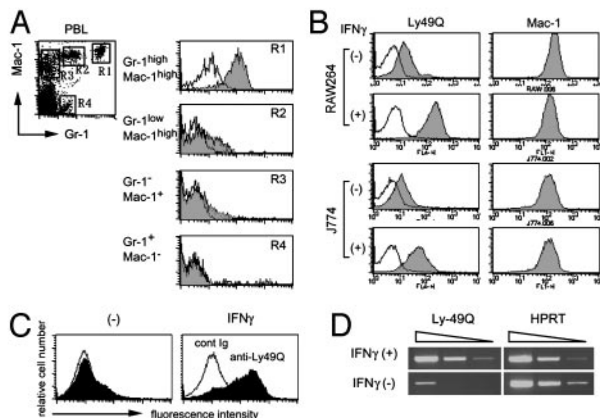

Fig. 5. Expression of Ly49Q on macrophages. (*A*) Flow cytometry of MNCs from peripheral blood leukocytes stained with FITC-conjugated anti-Mac-1, PE-conjugated anti-Gr-1, and biotin-conjugated anti-Ly49Q mAb (shaded histograms), followed by staining with allophycocyanin-conjugated streptavidin. Control stainings with isotype-matched rat IgG2a were overlaid in each histogram (open histograms). (*B*) Flow cytometry of mouse macrophage cell lines RAW264 and J774 treated with or without 50 units/ml IFN-γ for 24 h and stained with anti-Ly49Q mAb (*Left*) or anti-Mac-1 mAb (*Right*). (*C*) Flow cytometry of TGC-PEC cultured with or without IFN-γ for 48 h and stained with anti-Ly49Q mAb (shaded histograms) or control Ab (open histograms). (*D*) RT-PCR with the primers specific for either Ly49Q or hypoxanthine phosphoribosyltransferase (HPRT) RNA prepared from J774 treated with or without IFN-γ.

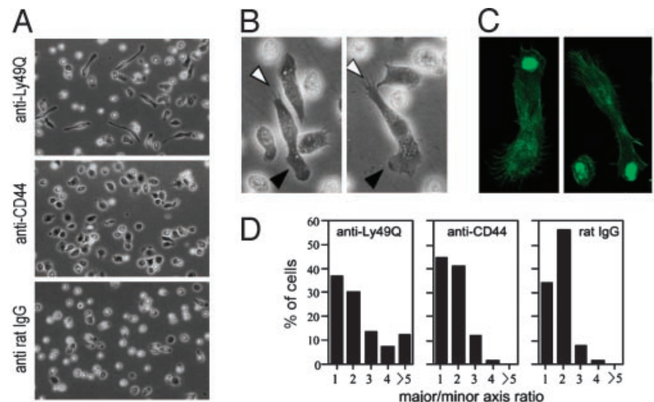

Fig. 6. Effects of anti-Ly49Q mAb on adhesiveness and spreading of macrophages. (*A*) Photomicrographs of RAW cells plated on Falcon six-well plates coated with 20 μg/ml anti-Ly49Q mAb, anti-CD44 mAb, or control rat IgG, and incubated at 37°C for 15 min. (*B*) Photomicrographs of RAW cells spread on wells coated with anti-Ly49Q mAb shown at higher magnification. Filopodia-like structures are indicated by white arrowheads, and lamellipodia-like structures are indicated by shaded arrowheads. (*C*) Fluorescence micrograph of actin filaments of RAW cells spread on wells coated with anti-Ly49Q mAb stained with rodamine-conjugated phalloidin. (*D*) Measurements of RAW cells plated on antibody-coated wells and incubated at 37°C for 20 min. The major/minor axis ratio of photographed cells was determined. Histograms represent the frequency in each class intervals of major/minor axis ratio. (Magnifications: $\times$200, *A*; $\times$600, *B* and *C*.)

monocytes/macrophages (30). These results suggest that immature myeloid precursors express high levels of Ly49Q, and the expression decreases as they mature. As shown in Fig. 5*B*, two murine macrophage cell lines, RAW264 and J774, expressed low, but significant, levels of Ly49Q. Interestingly, treatment of these cell lines with IFN-γ induced up-regulation of Ly49Q expression (Fig. 5*B*). Similar results were obtained when peritoneal macrophages were used (Fig. 5*C*). The enhanced expression of Ly49Q was also detected at the mRNA level (Fig. 5*D*). These results indicate that expression of Ly49Q is up-regulated during macrophage activation by IFN-γ.

Rapid Change of Cell Shape and Reorganization of Cytoskeleton by Anti-Ly49Q mAb. We next investigated whether Ly49Q was involved in regulation of macrophage functions. Although treatment of macrophages with NS 34 did not affect production of IL-12, nitrite oxide, or phagocytosis in our assay conditions (data not shown), we discovered that contact with the mAb induced a rapid, striking morphological change in the cells under certain conditions. When Ly49Q-expressing macrophage cell line RAW264 was plated on a NS 34-coated plate, the cells rapidly adhered and elongated on the plate (Fig. 6*A*). The elongated cells tended to be polarized as shown in Fig. 6 *B* and *C* with lamellipodia at one end and filopodia at the opposite end of the cells. Cell spreading was observed within 10 min after cells were added to NS 34-coated wells and increased with further incubation. The frequency of highly spread cells with length-to-width ratios >4 reached 16% in mAb-treated cells (Fig. 6*D*). This frequency was significantly higher than the $<1\%$ elongated cells plated on control rat IgG-coated or isotype-matched anti-CD44 mAb-coated wells.

Enhanced cell spreading was also observed when thioglycollate (TGC)-induced peritoneal macrophages were put on plates treated with NS 34; no spreading was observed with control Abs (Fig. 7*A*). Such morphological change was detectable only when cells were prestimulated with IFN-γ. Interestingly, some TGC-induced peritoneal macrophages, as shown in Fig. 7*B*, protruded microscopically dense, ring-like structures known as podosomes

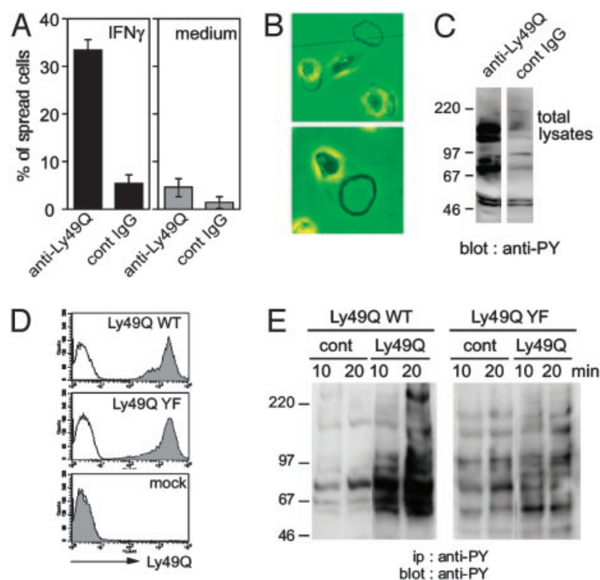

Fig. 7. Signal transduction by crosslinking of Ly49Q molecules by anti-Ly49Q mAb. (*A*) Spreading efficiency of TGC-PEC treated with (*Left*) or without (*Right*) IFN-γ for 48 h and plated on antibodies-coated dishes after a 10-min incubation. Cells were photographed, and images were counted. (*B*) Photographs of TGC-PEC spreading on anti-Ly49Q-coated dishes. (Magnification: ×600.) (*C*) Western blot analysis of tyrosine-phosphorylated proteins in IFN-γ-treated TGC-PEC plated on culture dishes coated with either anti-Ly49Q mAb or control rat IgG, incubated at 37°C for 10 min. (*D*) Cytometry of WEHI3 cells infected with retrovirus encoding Ly49Q WT cDNA, Ly49Q YF cDNA, or mock cDNA, and GFP-positive cells were sorted by FACSVantage. WEHI3 transfectants were stained with either anti-Ly49Q mAb (shaded histograms) or control rat IgG (open histograms). (*E*) WEHI3 transfectants treated with IFN-γ and lipopolysaccharide were plated on culture dishes coated with either anti-Ly49Q mAb or control rat IgG and incubated at 37°C for indicated periods. Tyrosine-phosphorylated proteins were precipitated with 4G10-conjugated agarose beads followed by immunoblotting with 4G10 mAb.

(31). Other anti-Ly49Q mAb NS 2-2 also induced morphological changes in IFNγ-treated J774 and peritoneal macrophages (data not shown). Thus, in all macrophage cells used, triggering of Ly49Q molecules induced rapid spreading and formation of cell polarity through reorganization of actin cytoskeleton.

Induction of Tyrosine Phosphorylation of Various Proteins After Crosslink of Ly49Q with Specific mAb. A panel of proteins became tyrosine-phosphorylated when TGC-induced peritoneal macrophages were plated on Ly49Q mAb-coated plate but not on control rat IgG-coated plate (Fig. 7*C*). To further examine Ly49Q-mediated signal transduction, Ly49Q transfectants were established from the WEHI3 myelomonocytic cell line that does not express endogenous Ly49Q on their surface (Fig. 7*D*). Biochemical features of Ly49Q expressed in WEHI3 cells were almost the same as those in COS7 cells. Tyrosine phosphorylation-dependent association of SHP-1 and SHP-2 was also observed in WEHI3 cells (data not shown). A substantial increase in overall protein-tyrosine phosphorylation was observed on anti-Ly49Q-coated plates, but phosphorylation did not increase when cells were placed on control rat IgG-coated plates (Fig. 7*E*). In contrast, the induction of tyrosine phosphorylation was not observed in the case of Ly49Q YF-transfected WEHI3 cells, indicating that the tyrosine residue in the ITIM is essential in signal transduction through Ly49Q.

Discussion

In the present study, we identified and characterized a member of the Ly49 family, Ly49Q. It exhibits a number of unique features for a member of the family: (*i*) Expression of Ly49Q could not be detected on NK and NKT cells. (*ii*) Ly49Q is expressed on the surface of Gr-1$^+$ cells in fetal liver, bone marrow, spleen, and peripheral blood. (*iii*) The expression of Ly49Q on macrophages is up-regulated by IFN-γ. (*iv*) Plating of macrophages on anti-Ly49Q mAb-coated surfaces induced rapid attachment and elongation of the cells with the extension of polarized pseudopods.

It has been reported that myeloid lineage cells express various ITIM-bearing inhibitory receptors such as ILT family members in humans and the rodent orthologues, the PIRs (15, 32, 33). ILT2 and ILT4 recognize a broad range of MHC class I molecules and transduce an inhibitory signal (34, 35). PIR-B has been shown to be involved in inhibition of mast cell activation through associated SHP-1 (2, 36). Although there has been much debate about the functional significance and the ligands of these molecules, these receptors seem to play important roles to modulate cellular responses to external stimuli and maintain a homeostatic balance. Ly49Q may, as do other ITIM-bearing receptors on myeloid lineage cells, play an inhibitory function on these cells.

Ly49Q is the only known example of a Ly49 family member that is not expressed on NK and NKT cells. IL-2 stimulation of NK and NKT cells fails to induce expression of Ly49Q on their surface (data not shown). Thus, Ly49Q may not function as an NK cell receptor. Instead, expression of Ly49Q appears to be confined to myeloid lineage cells, such as myeloid precursor cells in bone marrow and activated macrophages. Monocytes, resting macrophages, and neutrophils in peripheral blood express little or no Ly49Q. Expression of Ly49Q on macrophages is up-regulated within 24 h after the addition of IFN-γ. It seems likely that expression of Ly49Q decreases as these cells mature, and that mature macrophages re-express Ly49Q at an early time point of the inflammatory responses in the presence of IFN-γ.

Flow cytometric analysis revealed that the vast majority of Ly49Q$^+$ cells in bone marrow express Gr-1. In our RT-PCR analysis, some Gr-1$^-$ cells in bone marrow also seem to transcribe Ly49Q mRNA. This finding might be caused by the presence of a Gr-1^{dull} population in the Gr-1$^-$ fraction. Alternatively, some Gr-1$^-$ cells in bone marrow may express high levels of Ly49 mRNA. Indeed, a small portion of Gr-1$^-$ cells appears to be stained with anti-Ly49Q mAb (Fig. 3*E*). These cells do not express surface markers of NK, NKT, and T cells (data not shown) but express B220, supporting the notion that Ly49Q is not expressed on NK and NKT cells. Further analysis is needed to determine the lineage of these cells.

Plating of macrophages on a surface coated with anti-Ly49Q mAb rapidly induced attachment, elongation, and the polarized formation of lamellipodium and filopodia in opposite directions. In the case of TGC-induced peritoneal macrophages, prior IFN-γ treatment was necessary for the induction of morphological change. Because IFN-γ induces up-regulation of Ly49Q expression on macrophages, higher expression of Ly49Q on the cell surface may be necessary for Ly49Q-mediated morphologic change. In RAW264 cells, polarized protrusion of a filopodia-like structure was also observed opposite a lamellipodium similar to the migration phenotype of macrophages. It is notable again that these morphologic changes are rapid and occur within a few minutes after the cells are exposed to the mAb. This mechanism may be important in rapid mobilization of cells *in vivo*. Thus Ly49Q, as are chemokines, may be involved in regulation of rapid cell movement *in vivo*.

Anti-CD44 mAb with the same isotype as NS 34 failed to elicit polarized cell spreading even though it reacts with CD44 expressed on the macrophage cell surfaces. This finding suggests that polarized spreading is not caused by nonspecific binding of Abs to Fc receptors or to macrophage cell surface molecules. There are at least two possibilities for the mechanisms by which

the mAbs induce changes in the macrophages. Anti-Ly49Q mAbs could inhibit binding of a ligand or mimic binding of a ligand, resulting in transduction of signals through Ly49Q. When anti-Ly49Q mAb NS 34 is added to macrophages in suspension, marked cell polarization or cell attachment is not observed (data not shown). Immobilization of the mAb on culture dishes is necessary to induce spreading. This finding suggests that crosslinking of Ly49Q by the mAb may be necessary for induction of polarized spreading and that anti-Ly49Q mAb NS 34 mimics a ligand binding to Ly49Q.

Increase in tyrosine-phosphorylated proteins in TGC-induced peritoneal macrophages and accelerating spreading of macrophages by the treatment with anti-Ly49Q mAb implies that Ly49Q introduces activation signals rather than inhibitory signals even though it has an ITIM. The KIR2DL4 ITIM-bearing receptor in humans has been shown to produce activating signals by association of DAP10 and DAP12 with a charged transmembrane domain of these receptors (37). Because Ly49Q does not contain a charged amino acid residue essential for the association with DAP10 and DAP12 in its transmembrane region, it is unlikely that Ly49Q transduces signals through these signaling molecules (38, 39).

Our immunoprecipitation and Western blot analysis using COS7 and WEHI3 cells expressing Ly49Q WT and YF mutants clearly indicate that Ly49Q can engage both SHP-1 and SHP-2, and that the association of Ly49Q with these phosphatases depends on the tyrosine residue in the ITIM. In addition, biochemical analysis using WEHI3 transfectants indicates that the tyrosine residue in the ITIM is essential for transducing signals through Ly49Q into cells. Taken together, our results suggest that ITIM-associated SHP-1 or SHP-2 is involved in signaling by Ly49Q, although the mechanisms of Ly49Q-mediated rapid spreading still remain unclear. SHP-1 and SHP-2 are highly homologous, and a number of receptors with an ITIM engaged both SHP-1 and SHP-2 (11–13). We cannot state which phosphatases may preferentially associate with Ly49Q in macrophages. Immunoprecipitation of endogenous Ly49Q from macrophages with NS 34 was not successful, likely because NS 34 mAb has a lower ability to precipitate Ly49Q than anti-FLAG mAb has (Fig. 3*B*).

It is important to identify a ligand for Ly49Q to understand the physiological function of Ly49Q and polarized spreading. Conventional Ly49 family members expressed on NK and NKT cells recognize MHC class I and its related molecules. Although a ligand of Ly49Q is still not identified, our finding raises an intriguing possibility that macrophage function is regulated by Ly49Q in self and nonself discrimination.

Our finding that cytoskeletal architectures of macrophages could be regulated by Ly49Q is particularly interesting because cytoskeleton in macrophages is crucial for migration and phagocytosis. Up-regulated expression of Ly49Q on macrophages by IFN-γ may allow rapid movement of macrophages to survey and ingest microbial components in inflamed tissues. Further investigation of Ly49Q could reveal new roles for inhibitory receptors in regulation of cytoskeletal architectures and cell migration.

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