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Ly49Q Positively Regulates Type I IFN Production by Plasmacytoid Dendritic Cells in an Immunoreceptor Tyrosine–Based Inhibitory Motif–Dependent Manner

Mir Munir A. Rahim,*1 Lee-Hwa Tai,1 Angela D. Troke,* Ahmad Bakur Mahmoud,*,‡ Elias Abou-Samra,* Justin G. Roy,* Amelia Mottashed,* Nicholas Ault,* Chloe Corbeil,* Marie-Line Goulet,* Haggag S. Zein,*,§ Melissa Hamilton-Valensky,* Gerald Krystal,* William G. Kerr,‖# Noriko Toyama-Sorimachi,** and Andrew P. Makrigiannis*

Plasmacytoid dendritic cells (pDC) are the major producers of type I IFN during the initial immune response to viral infection. Ly49Q, a C-type lectin-like receptor specific for MHC-I, possesses a cytoplasmic ITIM and is highly expressed on murine pDC. Using Ly49Q-deficient mice, we show that, regardless of strain background, this receptor is required for maximum IFN-α production by pDC. Furthermore, Ly49Q expression on pDC, but not myeloid dendritic cells, is necessary for optimal IL-12 secretion, MHC-II expression, activation of CD4+ T cell proliferation, and nuclear translocation of the master IFN-α regulator IFN regulatory factor 7 in response to TLR9 agonists. In contrast, the absence of Ly49Q did not affect plasmacytoid dendritic cell–triggering receptor expressed on myeloid cells or pDC viability. Genetic complementation revealed that IFN-α production by pDC is dependent on an intact tyrosine residue in the Ly49Q cytoplasmic ITIM. However, pharmacological inhibitors of protein tyrosine phosphatases and phosphatase-deficient mice indicate that Src homology 2 domain-containing phosphatase 1 (SHP)-1, SHP-2, and SHIP phosphatase activity is dispensable for this function. Finally, we observed that Ly49Q itself is downregulated on pDC in response to CpG exposure in an ITIM-independent manner. In conclusion, Ly49Q enhances TLR9-mediated signaling events, leading to IFN regulatory factor 7 nuclear translocation and expression of IFN-I genes in an ITIM-dependent manner that can proceed without the involvement of SHP-1, SHP-2, and SHIP. The Journal of Immunology, 2013, 190: 3994–4004.

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lasmacyotid dendritic cells (pDC) are a subset of dendritic cells (DC) that are specialized in direct virus recognition and secretion of large amounts of type I IFN (IFN-I) (1).

*Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada; ‡Centre for Innovative Cancer Research, Ottawa Hospital Research Institute, Ottawa, Ontario K1Y 4E9, Canada; †College of Applied Medical Sciences, Taibah University, 30001, Madinah Munawwarah, Kingdom of Saudi Arabia; ‡Cairo University Research Park, Faculty of Agriculture, Cairo University, Giza 12613, Egypt; ‣Terry Fox Laboratory, BC Cancer Agency, Vancouver, British Columbia V5Z 1L3, Canada; ‥Department of Pediatrics, State University of New York Upstate Medical University, Syracuse, NY 13210; ‡Department of Microbiology and Immunology, State University of New York Upstate Medical University, Syracuse, NY 13210; and **Department of Molecular Immunology and Inflammation Research Institute, National Center for Global Health and Medicine, Tokyo 162-8655, Japan

M.M.A.R. and L.-H.T. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Andrew P. Makrigiannis, Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Roger Guindon Hall, Room 4226, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada. E-mail address: amakr1g@uottawa.ca

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; BST2, bone marrow stromal Ag 2; DC, dendritic cell; DOTAP, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-n-palmitate; ILT7, Ig-like transcript 7; IRF, IFN regulatory factor; KIR, killer Ig-like receptor; mDC, myeloid dendritic cell; NKT, natural killer T cell; PDC, plasmacytoid dendritic cell; PDC–TREM, PDC-triggering receptor expressed on myeloid cells; PTP, protein tyrosine phosphatase; SH2, Src homology 2; SHIP, SH2 domain-containing phosphatase 1; SSG, sodium stibogluconate; Tg, transgenic; WT, wild-type.

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PDC represent <1% of lymphoid organ cells and are primarily located in the T cell areas of the spleen, lymph nodes, and Peyer’s patches (1, 2). They can secrete up to 1000 times more IFN-I than other cell types in response to microbial challenge (3). This property of pDC makes them an important component of the innate immune response against viruses. In addition, murine pDC can secrete IL-12, IL-6, and TNF-α, and acquire the ability to directly stimulate T cell proliferation (4, 5). Ablation of pDC in mice leads to enhanced viral burden and impaired virus-specific cytotoxic T cell responses (5). pDC detect the presence of microbial nucleic acids through TLRs, which are a subset of pattern recognition receptors that recognize pathogen-associated molecular patterns. pDC selectively express TLR7 and TLR9, which recognize ssRNA and unmethylated CpG dinucleotides of dsDNA, respectively (6).

Ly49Q is a member of the large Ly49 family of type II integral membrane receptors with extracellular C-type lectin domains (7). Genes encoding Ly49 proteins, termed Kira, are clustered together in the NK gene complex on mouse chromosome 6, and the gene encoding Ly49Q is part of a set of framework genes that are conserved in all known mouse Ly49 haplotypes, whereas other Ly49 genes are highly polymorphic and present in variable numbers (8). With the exception of Ly49Q, Ly49B, and Ly49E, all other Ly49 family members are primarily expressed on NK and NKT cells (9, 10). Ly49Q is expressed on Gr1+ myeloid lineage cells in the bone marrow, spleen, and peripheral blood in all strains of mice tested (7, 11). Virtually all pDC in blood, lymph nodes, spleen, and a subset of pDC in the bone marrow express Ly49Q (12). Furthermore, Ly49Q expression on pDC correlates with sequential development and activation (11–13). In macrophages,
Ly49Q expression is increased by IFN-γ–induced activation, and its cross-linking induces cytoskeletal rearrangement (7). Similarly, Ly49Q regulates neutrophil polarization and migration to sites of inflammation (14). Ly49Q also regulates osteoclast differentiation in vitro (15).

Colorimetric reporter cell assay and class I MHC (MHC-I) tetramer-binding analyses have revealed the MHC-I H-2Kb molecule as a ligand for Ly49Q (16). Experiments with Ly49Q-deficient mice as well as pDC from wild-type (WT) mice have shown that, despite the presence of an ITIM in the cytoplasmic domain of Ly49Q, it is stimulatory in nature and required for TLR-mediated IFN-I production by pDC (17). Specifically, the IFN-α and IL-12 produced by ex vivo pDC stimulated with CpG oligodeoxynucleotide (ODN) can be blocked with soluble mAb to Ly49Q or H-2Kb. In contrast, receptor cross-linking with plate-bound anti-Ly49Q or with recombinant H-2Kb increased cytokine production (17). In line with these observations, pDC isolated from Ly49Q-deficient mice displayed a significant defect in IFN-α and IL-12 secretion in response to CpG ODN or influenza virus compared with WT pDC (17). Furthermore, sera obtained from CpG- injected Ly49Q-deficient mice displayed reduced IFN-α levels. In contrast, TNF-α and IL-6 production was not compromised in Ly49Q-deficient pDC (17). Thus, despite the presence of an ITIM in its cytoplasmic domain, the Ly49Q receptor is stimulatory in nature and required for TLR-mediated IFN-I production by pDC. In contrast, receptors that signal through ITAM-mediated pathways negatively regulate cytokine production by pDC. These receptors include Ig-like transcript 7 (ILT7), bone marrow stromal Ag 2 (BST2; BDCA-2; CD317), Nkp44 on human pDC, and SiglecH on mouse pDC, all of which signal through ITAM-containing adapters such as DAP12 and FcεRIγ (18–20). Accordingly, DAP12-deficient pDC display increased cytokine production in response to viral infections (21). However, an exception is PDC-triggering receptor expressed on myeloid cells (PDC-TREM), a member of the triggering receptor expressed on the myeloid cell family, which is expressed on activated pDC and augments IFN-I production in a DAP12-dependent manner (22).

In a follow-up study, we demonstrated a novel mechanism by which TLR9 signaling is controlled through the spatiotemporal regulation of membrane trafficking by the ITIM-bearing receptor Ly49Q. Macrophages and pDC lacking Ly49Q showed altered distribution of TLR9 and CpG ODN (23). In particular, CpG ODN–induced tubular endolysosomal extension was impaired in the absence of Ly49Q. Consistent with these findings, cells lacking Ly49Q showed impaired cytokine production in response to CpG ODN (13).

Protein tyrosine phosphatases (PTPs) play an integral role in the regulation of immune cell activation. Immune cells express a remarkably large number of PTPs, highlighting their importance in immune cell function (24). A number of inhibitory immune cell receptors, including human killer Ig-like receptor (KIR) and mouse Ly49 NK receptors, possess ITIMs that, when phosphorylated, bind to Src homology 2 (SH2) domain-bearing PTPs (25). The majority of these PTPs inhibit activation of immune cells (24). Human KIR and mouse Ly49 ITIMs, upon phosphorylation, recruit SH2 domain-containing phosphatase 1 (SHP-1) and SHP-2 to inhibit NK cells (26–28). Some Ly49 can also recruit SHIP through their ITIM (29). Ly49B is expressed on a subpopulation of myeloid cells and is shown to associate with SHP-1, SHP-2, and SHIP-1 in transfected cells (9). SHP-1 plays an important role in the regulation of cytokine production. Paradoxically, whereas SHP-1 negatively regulates production of proinflammatory cytokines in lymphocytes, it has activating function in the production of IFN-I, thus contributing to immune homeostasis (30). Similarly, Ly49Q has been shown to recruit SHP-1 and SHP-2 in an ITIM phosphorylation-dependent manner (7). We have shown the significance of Ly49Q phosphatase recruitment in neutrophil studies. In the steady state, Ly49Q inhibits neutrophil adhesion by preventing focal-complex formation. However, in the presence of inflammatory stimuli, Ly49Q mediates rapid neutrophil polarization and tissue infiltration in an ITIM domain–dependent manner (14). These functions appear to be mediated by distinct use of the effector phosphatases SHP-1 and SHP-2. Ly49Q-dependent neutrophil polarization and migration were affected by Ly49Q regulation of membrane raft functions (14). However, the significance of phosphatase recruitment in pDC function is unknown.

Members of the IFN regulatory factor (IRF) family of transcription factors are critical for induction of the IFN-I response (31). Specifically, IRF3 and IRF7 enhance IFN-I mRNA expression when expressed ectopically (32, 33). IRF7 expression is induced by IFN-α and regulates both late-phase induction as well as positive feedback induction of the IFN-α response (34). In contrast, IRF3 is constitutively expressed and regulates IFN-β and the early phase of IFN-α induction (32). Hence, IRF3 and IRF7 perform distinct nonredundant functions in the induction of IFN-I genes (35). IRF7 is critical for both virus-activated MyD88-independent as well as TLR-activated MyD88-dependent induction of IFN-I (36). In pDC, TLR9-mediated IFN-I production is dependent on IRF7 (36).

The present study investigates the mechanism of Ly49Q augmentation of the TLR-mediated IFN-I response by pDC. We demonstrate that the defect in IFN-α production by pDC from Ly49Q-deficient mice is restored by expression of WT Ly49Q, but not by a Ly49QYF mutant in which the tyrosine residue in the ITIM has been mutated to phenylalanine. These findings confirm the critical function of Ly49Q in the regulation of IFN-α production by pDC, and that this mechanism of cytokine induction is dependent on the cytoplasmic domain ITIM of Ly49Q. We also show that Ly49Q positive regulation of IFN-α production by pDC is mediated through activation and translocation of IRF3 to the nucleus. In addition, we investigated the role of SH2 domain–bearing phosphatases in this process.

Materials and Methods

**Mice**

Ly49Q-null mice were described previously (22). Ly49QWt and Ly49QYF transgenic (Tg) mice were generated as previously described (14). Viable motheaten (me3) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). SHIP−/− mice were described previously (29). These mice were bred with C57/6-Tg mice (The Jackson Laboratory) to generate SHIP−/− mice. All mice were maintained in a specific pathogen-free environment. All breeding and manipulations performed on animals were in accordance with university guidelines and approved by the University of Ottawa Animal Ethics Committee.

**Flow cytometry**

The following Abs were obtained from commercial sources: allophycocyanin–mPDCA-1 (anti–B220; Miltenyi Biotec), FITC–440c (anti–Siglech), biotin–MHCH, FITC–CD11c, allophycocyanin–CD45R (anti–B220) (eBioscience), FITC–2E6 (anti-Ly49Q) (MBL International), PE–PDC-TREM (BioLegend), and PE–mouse IL-12 (BD Pharmingen). PE–streptavidin and 7-aminoactinomycin D (7-AAD) were purchased from eBioscience. Biotin–NS34 (anti-Ly49Q) was generated as previously described (7). Splenocytes were prepared by injecting the spleens with collagenase-D (Roche). The injected spleens were minced and incubated in 5 ml collagenase-D (0.5 mg/ml) for 20 min at 37°C. EDTA (Life Technologies) was added at 1 μM concentration and incubated for additional 5 min to dislodge cells. Spleen fragments were crushed and passed through a 70-μm cell strainer (Fisher Scientific) to remove large debris. RBCs were lysed with ACK lysis buffer for 5 min at 4°C. Cells were washed and kept in cold PBS. For FACS analysis, cells were washed with FACS buffer (PBS, 0.5% BSA, 0.02% NaN3) and incubated with fluorochrome-conjugated Abs.
for 20 min at 4°C. For secondary staining, cells were washed in FACS buffer and further incubated with the appropriate Abs for 20 min at 4°C. For intracellular staining, cells were fixed with Cytofix/Cytoperm reagent (BD Biosciences) and stained following manufacturer’s instructions. To stain dead/apoptotic cells, 7-AAD was added at 0.25 μg/10^6 cells and incubated for 5 min at room temperature. Cells were finally washed in FACS buffer and analyzed with a CyAn flow cytometer and Kaluza software (Beckman Coulter).

**In vivo pDC stimulation**

Mice were injected i.v. through the tail vein with 10 μg CpG-B (ODN 2006) or CpG-A DNA (ODN 2216) (Hycluc Biotech) and 1.2-dioleoylxylo-3-trimethylammonium-propane (DOTAP; Roche) preparation (170 μg CpG ODN diluted in sterile PBS plus 30 μl DOTAP). Serum was prepared from blood obtained through the cheek vein 6 h after CpG ODN injection. Serum cytokine levels were analyzed by ELISA, as previously described (17), or FlowCytomix bead assay (eBioscience) following the manufacturer’s instructions.

**In vitro pDC and DC2.4 stimulation**

pDC were isolated from spleens using an AutoMACS-pro instrument (Miltenyi Biotec) and pDC isolation kit-II (Miltenyi Biotec), according to the manufacturer’s protocol. Isolated pDC were seeded at 10^5 cells/well in a flat-bottom 96-well plate in 200 μl pDC medium (RPMI 1640, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.05 mM L-glutamine, and 55 μM 2-ME) for 16 h at 37°C and 5% CO2. CpG ODN, at the indicated concentrations, and sodium sibogulcan (SSG, 10 or 100 μg/ml; Calbiochem) were added in the corresponding wells. Culture supernatant was collected and stored at -80°C for analysis with ELISA or FlowCytomix bead assay. For surface Ly49Q analysis, isolated pDC were treated with CpG-A, CpG-B ODN (10 μg/ml), or LPS (1 μg/ml), as above, for 4 h. The DC2.4 cell line was transfected with Ly49Q (B6 allele) or ITIM-mutated Ly49QisoF using lipofectamine transfection reagent (Invitrogen), and stable clones were selected. Cells were treated with CpG ODN (10 μg/ml), LPS (1 μg/ml), poly(I:C) (15 μg/ml), or imiquimod (3 μg/ml) in vitro for 4 h. Ly49Q and MHC-I expression was analyzed by flow cytometry.

**Immunofluorescent staining and confocal microscopy**

In vivo stimulated pDC were isolated with an AutoMACS-pro instrument, as described above. pDC were resuspended in PBS and cytosptin onto glass slides (Fisher Scientific) at 800 rpm for 3 min. Slides were allowed to dry overnight and fixed in methanol for 10 min at -20°C. Following three washes with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. Slides were washed three times with PBS and blocked with 5% BSA in PBS containing 0.2% Tween 20 (PBS-T) for 1 h at room temperature. Slides were then incubated with a 1:250 dilution of rabbit polyclonal Ab against mouse IRF7 (Abcam) in blocking buffer for 2 h at room temperature. After three washes with PBS-T, slides were incubated with a 1:500 dilution of Alexa-488–conjugated anti-rabbit IgG Ab (Invitrogen) in PBS-T with 3% BSA for 45 min at room temperature. Slides were then washed with PBS-T and incubated with DAPI (Invitrogen) at 1 μM/ml in PBS for 10 min at room temperature. Finally, slides were washed three times in PBS and mounted with a coverslip using Mowiol 4-88 mounting medium (Calbiochem) following the manufacturer’s instructions. After setting for 24 h at 4°C, confocal images were acquired with a laser-scanning microscope LSM 510 META (Zeiss) using the ×40 oil-emersion objective.

**IFN-α measurement**

Mouse IFN-α concentration in culture supernatant and serum was determined by sandwich ELISA, as described previously (17), or by FlowCytomix bead assay (eBioscience). For ELISA, samples were analyzed at 1:10 dilution and OD was read at 450 nm with a PowerWave XS2 microplate reader (BioTek). FlowCytomix bead assay was performed as per the manufacturer’s instructions and analyzed with a CyAN flow cytometer (Beckman Coulter) and FlowCytomix Pro 2.2 software (eBioscience).

**Western blot analysis**

To increase pDC numbers, mice were injected s.c. with B16-F1H3L cells (a generous gift of S. Vidal, McGill University, Montreal, Montréal, QC, Canada), as described elsewhere (37). pDC were isolated from spleens with an AutoMACS-pro instrument, as described above. Proteins were extracted from isolated pDC using radioimmunoprecipitation assay buffer that contained 50 mM Tris (pH 8), 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40 detergent, and 0.1% SDS in presence of protease inhibitor mixture (Calbiochem). The amount of protein in the lysate was determined by Bio-Rad protein assay reagent (Bio-Rad), as per the manufacturer’s instructions. Protein samples were resolved on SDS-PAGE and transferred to a membrane. The membranes were blotted with Abs against IRF7 (Abcam), GAPDH (Abcam), SHP-1, SHP-2, and SHIP (generous gifts of A. Veillette, Institut de Recherches Clinique de Montreal, Montréal, QC, Canada). Signals were detected with HRP-conjugated secondary Ab and ECL Plus Western blot detection system (Amersham).

**Results**

**Defective TLR9-mediated IFN-α production by Ly49Q-deficient pDC on the B6 genetic background**

We have previously shown a profound defect in TLR9-mediated IFN-α production by pDC lacking Ly49Q (17). For the purpose of the current study, the Ly49Q-deficient mice, which were originally generated on the 129S1 genetic background, were bred onto the C57BL/6 (B6) genetic background for 12 generations. These mice are essentially congenic for the region of mouse chromosome 6, which contains the Kira (Ly49) gene cluster and the disrupted Ly49q1 gene from 129-strain mice (data not shown). B6.Ly49q129B congenic mice, which have the 129S1-derived Ly49q1 gene cluster on a B6 genetic background, were used as WT controls (38). Mouse strain-specific differences in secretion of IFN-α by pDC are well documented. In particular, 129-strain mice produce higher levels of IFN-α than B6 mice when challenged with CpG ODN (2). The production of IFN-α after systemic stimulation by CpG ODN is due solely to pDC, as shown by IFN-α enhanced GFP knock-in reporter mice (39).

To determine whether the strain-specific differences in IFN-α production are due to Ly49 haplotype variation, splenic pDC were isolated from B6, 129S1, and B6.Ly49q129B congenic mice by negative selection and stimulated overnight with CpG-B ODN. IFN-α levels were analyzed in the culture supernatant by ELISA. As expected, B6 pDC produced significantly lower levels of IFN-α as compared with 129S1 pDC (Fig. 1A). pDC from B6.Ly49q129B congenic mice produced IFN-α levels similar to that of B6 mice (Fig. 1A), suggesting that the background of the NK gene complex genes, including Ly49, does not affect pDC IFN-α production. To determine IFN-α production capacity in Ly49Q-deficient mice on a B6 background, B6.Ly49q129B congenic (WT) and Ly49q-deficient mice were injected with CpG ODN i.v., and serum IFN-α levels were evaluated after 6 h. As expected, CpG-A-ODN induced higher serum IFN-α levels than CpG-B ODN treatment in WT mice. Serum IFN-α levels were significantly lower in Ly49Q-deficient compared with the WT mice when treated with CpG-A as well as CpG-B-ODNs (Fig. 1B). We also compared IFN-α production capacity of pDC isolated from Ly49Q-deficient mice on B6 background when stimulated overnight with various concentrations of CpG ODN in vitro. Ly49Q-deficient pDC consistently produced significantly reduced levels of IFN-α as compared with the pDC from WT control mice at various concentrations of CpG-B ODN (Fig. 1C). A similar defect in IFN-α production by Ly49Q-deficient pDC was observed when stimulated with CpG-A...
ODN at low concentrations (Fig. 1D). At higher concentration (10 μg/ml), CpG-A ODN was able to induce significant levels of IFN-α production by Ly49Q-deficient pDC in vitro, almost approaching the levels produced by WT pDC (Fig. 1D). These data together confirm our previous finding that Ly49Q positively regulates TLR9-mediated IFN-α production by pDC. Furthermore, this function is conserved in mice of different genetic backgrounds and with different CpG ODN types. Because CpG-B ODN consistently induce good levels of IFN-α production by WT and not Ly49Q-deficient pDC, in vitro as well as in vivo, it was used for the remainder of this study.

Defective IL-12 production and T cell stimulation by Ly49Q-deficient pDC, but not mDC

In addition to IFN-α, TLR9 stimulation also induces IL-12 production by murine pDC (4, 5). We previously reported lower serum IL-12 levels in Ly49Q positively regulates TLR9-mediated IFN-α production by pDC. Furthermore, this function is conserved in mice of different genetic backgrounds and with different CpG ODN types. Because CpG-B ODN consistently induce good levels of IFN-α production by WT and not Ly49Q-deficient pDC, in vitro as well as in vivo, it was used for the remainder of this study.

Defective IL-12 production and T cell stimulation by Ly49Q-deficient pDC, but not mDC

In addition to IFN-α, TLR9 stimulation also induces IL-12 production by murine pDC (4, 5). We previously reported lower serum IL-12 levels in Ly49Q-deficient compared with WT mice on the 129S1 background (17). To test IL-12 production by Ly49Q-deficient mice on B6 background, WT and Ly49Q-deficient mice were treated with CpG-B ODN in vivo for 6 h and flow cytometry analysis was performed to detect intracellular IL-12 in pDC and mDC ex vivo. Although CpG-B ODN treatment induced IL-12 production by WT pDC, it failed to do so in pDC from Ly49Q-deficient mice (Fig. 2A). In contrast, equal numbers of mDC from WT and Ly49Q-deficient mice stained positively for intracellular IL-12 (Fig. 2B). These data demonstrate a requirement for Ly49Q in TLR9-induced IL-12 production specifically in pDC and not mDC.

In vitro treatment with CpG-B ODN has been shown to induce pDC maturation and acquisition of the ability to stimulate T cell proliferation (40, 41). We measured class II MHC (MHC-II) levels on pDC from WT and Ly49Q-deficient mice treated with CpG-B ODN in vitro. MHC-II upregulation was almost 50% lower on Ly49Q-deficient pDC compared with WT pDC (Fig. 2C). To determine whether this corresponded to a lower T cell stimulation capacity in Ly49Q-deficient pDC, a mixed lymphocyte reaction was performed in which pDC or mDC from WT and Ly49Q-deficient mice were cultured with sorted BALB/c CD4+ T cells in the presence of CpG-B ODN, and T cell proliferation was measured by radioactive thymidine incorporation. In agreement with previous reports (42), the ability of pDC to stimulate T cells is much lower than mDC. Whereas mDC from both WT and Ly49Q-deficient mice induced equally high levels of CD4+ T cell proliferation, Ly49Q-deficient pDC were significantly less efficient in inducing CD4+ T cell proliferation compared with WT pDC when treated with CpG-B ODN in vitro (Fig. 2D). CpG-B ODN treatment alone did not induce proliferation on mDC, pDC, or CD4+ T cells in this assay. These data suggest that CpG-B ODN-induced maturation and acquisition of T cell proliferation capacity are disrupted in Ly49Q-deficient pDC.

Normal in vivo pDC activation and survival in Ly49Q-deficient mice

Reduced IFN-α production by Ly49Q-deficient pDC could be due to insufficient activation or survival of pDC lacking Ly49Q. pDC
activation was assessed by flow cytometric analysis of PDC-TREM expression after in vivo CpG-B ODN challenge. PDC-TREM is expressed on pDC activated by TLR7 and TLR9 ligands, and positively regulates IFN-α production (22). Ly49Q-deficient and WT mice were injected i.v. with CpG-B ODN, and PDC-TREM expression on pDC was analyzed 6 h later by flow cytometry. CpG-B ODN challenge induced expression of PDC-TREM on pDC. However, no difference in the percentage of pDC expressing PDC-TREM after CpG-B ODN challenge was observed between WT and Ly49Q-deficient mice (Fig. 3A), in agreement with prior studies of CD86 expression (17). These data suggest that the effect of Ly49Q on IFN-α production by pDC is independent of expression of activation markers on pDC.

pDC survival after in vivo CpG-B ODN challenge was assessed by 7-AAD staining and flow cytometry. Ly49Q-deficient and WT mice were injected i.v. with CpG-B ODN, and pDC were stained with 7-AAD after 6 h. CpG-B ODN challenge induced pDC cell death to the same level in WT and Ly49Q-deficient mice (Fig. 3B). This led to a similar decrease in splenic pDC numbers in WT and Ly49Q-deficient mice after CpG-B ODN challenge (Fig. 3C). As recently reported (43), pDC activation via TLR9 resulted in increased numbers of dying pDC. No significant differences in the frequency of apoptotic/dead pDC were observed prior to or after CpG-B ODN challenge between WT and Ly49Q-deficient mice. Together these results suggest normal activation and survival of pDC in the absence of Ly49Q expression.

**Essential role of the Ly49Q ITIM in TLR9-mediated IFN-α production by pDC**

Ly49Q possesses a cytoplasmic ITIM that is able to recruit phosphatases when phosphorylated (7). To determine the necessity of the Ly49Q ITIM in augmentation of TLR9-mediated IFN-α production by pDC, Ly49Q-deficient mice were bred with mice Tg for WT (Ly49Q WT) or ITIM-mutated (Ly49Q YF) Ly49Q in which...
the tyrosine residue in the ITIM is substituted with phenylalanine to generate Ly49Q/−/− mice and Ly49Q+/+ mice, respectively. Ly49Q expression on pDC was assessed ex vivo by flow cytometry and was found to be restored to normal levels on Ly49Q-deficient pDC carrying the Ly49Q WT or Ly49Q YF Tg (Fig. 4A).

To determine whether Ly49Q Tg expression restored IFN-α production in Ly49Q-deficient pDC, WT, Ly49Q-deficient, Ly49Q+/−, Ly49Q+/+ Tg mice on a B6 background were injected with CpG-B ODN i.v. and serum IFN-α levels were evaluated after 6 h. Expression of the Ly49Q WT Tg almost completely restored IFN-α production by Ly49Q-deficient pDC to the same level as the WT pDC (Fig. 4B). In contrast, the ITIM-mutated Ly49Q YF Tg expression failed to restore IFN-α production by Ly49Q-deficient pDC to the same level as the WT pDC (Fig. 4B). In contrast, the ITIM-mutated Ly49Q YF Tg expression failed to restore IFN-α production by Ly49Q-deficient pDC (Fig. 4B). Similar results were obtained with pDC isolated from WT, Ly49Q-deficient, Ly49Q+/−, Ly49Q+/+ Tg mice treated with CpG-B ODN in vitro (Fig. 4C). These data confirm the necessary role of Ly49Q in the regulation of IFN-α production by pDC.

Furthermore, the cytoplasmic ITIM of Ly49Q is shown to be critical for CpG-mediated IFN-α production by pDC.

Role of SH2 domain–bearing phosphatases in IFN-α production by pDC

The Ly49Q ITIM recruits SH2 domain–bearing phosphatase SHP-1 and SHP-2 when phosphorylated (7). Therefore, we investigated the possible role of these phosphatases in the regulation of IFN-α production by pDC. First, the expression of phosphatases in pDC was determined by Western blot analysis. To obtain enough pDC for this assay, B6.Ly49129 congenic mice were injected s.c. with B16-Flt3L cells, which have been shown to increase pDC numbers in mice (37). Western blot analysis of splenic pDC protein extracts showed the presence of SHP-1, SHP-2, and SHIP phosphatases in these cells (Fig. 5).

We next evaluated the role of these phosphatases in pDC IFN-α production with the use of SHP-1–mutant meV mice (44), as well as SSG, a chemical inhibitor of SHP-1 and SHP-2 (45). Splenic pDC were isolated from meV mice and put in culture overnight with various concentrations of CpG-B ODN. meV pDC produced similar levels of IFN-α as compared with WT pDC at all CpG-B ODN concentrations tested (Fig. 6A). This suggests that SHP-1

FIGURE 4. Ly49Q ITIM-dependent rescue of defective IFN-α production by Ly49Q-deficient pDC. Ly49Q/−/− mice were bred with Ly49Q+/+ Tg (Ly49Q+/−, QWT Tg) and Ly49Q YF Tg (Ly49Q+/−, QYF Tg) mice to restore Ly49Q expression on pDC. (A) Scatter plots represent flow cytometric analysis of Ly49Q surface expression on splenic pDC from the indicated mouse strains. Mean fluorescence intensity (MFI) is indicated. (B) Mice of the indicated genotypes were injected (i.v.) with CpG-B ODN + DOTAP. After 6 h, serum IFN-α levels were measured by ELISA. (C) IFN-α production by isolated pDC from mice of the indicated genotypes after CpG-B ODN stimulation in vitro. IFN-α levels were measured in culture supernatant by bead assay. The horizontal bar represents mean values. Each symbol represents the data from a single mouse. All p values are indicated.

FIGURE 5. Expression of SH2-bearing phosphatases in murine pDC. Western blot analysis of pDC protein extracts. Protein samples were resolved by SDS-PAGE and transferred to a membrane. The membrane was blotted with antisera against SHP-1 and SHIP (A), or SHP-2 (B) and detected by chemiluminescence. The membrane was stripped and blotted with Ab against GAPDH. Protein samples from cell lines (NIH3T3, 293T, and EL4) were used as controls.
deficiency alone does not disrupt TLR9-mediated IFN-α production by pDC. Similarly, when WT pDC were stimulated with CpG-B ODN in the presence of SSG at concentrations that inhibited SHP-1 specifically (10 μg/ml) or at concentrations that inhibit both SHP-1 and SHP-2 (100 μg/ml), no reduction in IFN-α production was observed (Fig. 6B). To evaluate the role of SHIP in pDC IFN-α production, splenic pDC were isolated from WT, ship+/−, and ship−/− mice and put in culture overnight with CpG-B ODN. SHIP-deficient pDC produced similar levels of IFN-α as compared with WT pDC (Fig. 6C). These experiments demonstrate that SHP-1, SHP-2, and SHIP are individually dispensable for pDC IFN-α production.

Ly49Q-induced augmentation of pDC IFN-α production is mediated through IRF7 transcription factor activation

Transcription factor IRF7 is critical for TLR9-mediated IFN-I production by pDC (36), and is constitutively expressed at high levels in human pDC (46). We confirmed that IRF7 is also constitutively expressed in mouse pDC at high levels by Western blot analysis of pDC protein extracts (Fig. 7A). To determine whether IRF7 is involved in Ly49Q-induced augmentation of IFN-α production by pDC after CpG-B ODN challenge, we assessed IRF7 activation and nuclear translocation by immunofluorescent staining and confocal microscopy. WT and Ly49Q-deficient mice were injected with CpG-B ODN i.v., and splenic pDC were isolated 6 h later. Immunofluorescent staining of IRF7 revealed nuclear translocation of IRF7 in WT mice, while fewer Ly49Q-deficient pDC showed translocation of IRF7 to the nucleus after CpG-B ODN challenge (Fig. 7B). These data suggest that the signaling events downstream of Ly49Q that regulate TLR9-mediated IFN-α production by pDC culminate in the activation of IRF7 and its translocation to the nucleus to induce IFN-α expression.

Evidence for a possible role of Ly49Q in CpG ODN trafficking

Our previous study has shown that TLR9 signaling is controlled through the spatiotemporal regulation of membrane trafficking by...
the ITIM-bearing receptor Ly49Q. In macrophages and pDC, TLR9 and CpG distribution is altered in the absence of Ly49Q (23). A recent study proposed a role for KIR3DL2 in uptake and trafficking of CpG ODN in NK cells (47). KIRs are the functional homologs of mouse Ly49 receptors on NK cells. KIR3DL2 was downmodulated from the NK cell surface and colocalized with CpG ODN in early endosomes where TLR9 is encountered. A direct binding of KIR3DL2 and CpG ODN was also demonstrated (47). To explore the role of Ly49Q in CpG ODN uptake and trafficking, we used DC2.4 cell line ectopically expressing Ly49QWT or ITIM-mutated Ly49Q YF on the cell surface. We observed a dramatic downmodulation of Ly49Q from cell surface in presence of CpG-A and CpG-B ODN (Fig. 8A, 8C). This downmodulation was seen as early as 2 h after CpG ODN treatment (data not shown). Similar downmodulation of ITIM-mutated Ly49Q YF was also observed in the presence of CpG ODN, indicating an ITIM-independent mechanism (Fig. 8A, 8C). Ly49Q was not downmodulated in presence of other TLR ligands such as LPS, poly(I:C), and imiquimod (Fig. 8B). In contrast to Ly49Q downmodulation, MHC-I surface expression on the same cells was unaffected in the presence of any of the TLR ligands tested (Fig. 8A, 8B). When isolated pDC from the BALB/c mouse strain were treated similarly, a slight but significant downmodulation of Ly49Q surface expression was observed with CpG-A and CpG-B ODN treatments, but not with LPS (Fig. 8C). These data suggest a possible role for Ly49Q in recognition and internalization of CpG ODN from the cell surface, which is independent of the ITIM. Further experiments are required to determine the nature of binding (direct versus indirect) and the role of Ly49Q and its ITIM in the trafficking of CpG ODN to early endosomes.

Discussion

We have previously shown that Ly49Q–MHC-I interaction positively regulates TLR-mediated cytokine production by pDC (17). Furthermore, we have reported that Ly49Q colocalizes with CpG ODN in the endosome/lysosome compartment of peritoneal macrophages and the murine macrophage cell line RAW264 (23). This colocalization appears to be crucial for intracellular trafficking of CpG ODN and TLR9 and cytokine production in response to CpG ODN (23). The present study confirms and extends these findings by demonstrating the critical function of the Ly49Q cytoplasmic domain ITIM in the regulation of IFN-α production by pDC.
through Tg complementation. Specifically, we observed a failure of Ly49Q<sup>T</sup> mutant receptor to restore IFN-α production in Ly49Q-deficient mice when stimulated with CpG-B ODN (Fig. 4B, 4C). WT Ly49Q transgene expression, in contrast, fully restored this function in Ly49Q-deficient mice. We also demonstrate that Ly49Q augmentation of the TLR-mediated IFN-I response by pDC is mediated through activation and translocation of IRF7 transcription factor to the nucleus (Fig. 7B). In addition, Ly49-deficient pDC fail to produce IL-12 (Fig. 2A) and failed to acquire the capacity to stimulate CD4<sup>+</sup> T cells in response to CpG-B ODN stimulation (Fig. 2D). However, pDC activation as measured by upregulation of PDC-TREM when stimulated with CpG-B ODN in vivo appears to be independent of IFN-α production and is unaffected by Ly49Q deficiency (Fig. 3A). Thus, unlike ITIM-bearing Ly49 receptors that impart inhibitory signals in NK cells, Ly49Q positively regulates TLR signaling in pDC. In line with this observation, receptors such as ILT7, BST2, Nkp44, and SiglecH, which signal through ITAM-containing DAP12 and FcεR1γ adaptor molecules and are expected to be stimulatory, negatively regulate cytokine production by pDC (19–21).

Ly49Q forms a framework gene that is conserved in all known mouse Ly49 haplotypes (8). Several alleles of Ly49Q have been reported, some of which contain polymorphisms in the ligand-binding domain, resulting in different binding affinities to MHC-I (11, 16). Our genetic studies with B6.Ly49<sup>290</sup> congenic mice, which have the 129S1-derived Ly49 gene cluster on B6 genetic background, demonstrate that the difference in the level of IFN-α production by different mouse strains is independent of strain origin of Ly49Q and the rest of Ly49 gene cluster. B6.Ly49<sup>290</sup> congenic pDC produce IFN-α to the same level as B6 pDC upon CpG-B ODN stimulation despite having 129S1-derived Ly49 gene cluster (Fig. 1A). Finally, we observed that Ly49Q positively regulates TLR9-mediated IFN-α production by pDC in two different genetic backgrounds, 129S1 and C57BL/6 (Fig. 1B–D), suggesting that this regulation mechanism is not a strain-specific phenomenon. Furthermore, this was evident with both CpG-A and CpG-B ODN stimulations. However, significant levels of IFN-α production were induced in Ly49Q-deficient pDC by higher concentrations of CpG-A ODN when stimulation was performed in vitro only (Fig. 1D). Guiducci et al. (41) have shown that the higher order structure (multimeric Cpg-A, monomeric Cpg-B) and endosomal location of Cpg ODNs influence pDC responses. We show evidence that Ly49Q may act as sensor for CpG ODNs and aid in their uptake and delivery to endosomal compartments (discussed below). The multimeric structure of Cpg-A ODN may allow its nonspecific uptake by pDC at high concentrations in vitro. Alternatively, other low-affinity DNA sensors may aid in uptake and shuttling of CpG-A ODN to endosomes in the absence of Ly49Q when high concentrations of CpG-A ODN are available. This is not seen with CpG-B ODN possibly due to its monomeric structure not being suitable for spontaneous uptake.

Ly49Q has been shown to recruit SHP-1 and SHP-2 in an ITIM phosphorylation-dependent manner (7), and SHP-1 deficiency results in reduced TLR-induced IFN-I production in total splenocytes (30). Furthermore, tyrosine phosphorylation plays an important role in mediating signals downstream of Ly49 receptor molecules. Inhibitory human KIR and mouse Ly49 NK receptors possess ITIMs that, when phosphorylated, bind to SH2 domain–bearing PTPs (25). Recruitment of SH2 domain–bearing PTPs SHP-1 and SHP-2 by KIR and Ly49 ITIMs results in inhibition of NK cell functions (26–28). Our studies with SHP-1–mutant me<sup>V</sup> mice and WT pDC stimulated with CpG-B ODN in the presence of inhibitor SSG did not show any significant defect in IFN-α production (Fig. 6A, 6B). Other SH2 domain–bearing phosphatases, such as SHIP, have been shown to be recruited by the phosphorylated ITIM of other Ly49 (29). IFN-α production by SHIP-deficient pDC is also normal in response to CpG-B ODN stimulation (Fig. 6C). In contrast to our results, An et al. (30) have shown that SHP-1 increases TLR-mediated production of IFN-I by inhibiting activation of kinase IRAK1. However, these experiments were carried out with total splenocytes, conventional DC, and bone marrow–derived DC. They also measured cytokine production by these cells in response to poly(I:C) and LPS, which are ligands for TLR3 and 4, respectively. PTPs regulate many aspects of immune cell functions and may have varied roles in different cell types (24). The discrepancy between the current study and the one by An et al. (30) can be attributed to the differences in the experimental designs. Similarly, SHP-2 has been shown to negatively regulate TLR3-mediated but not TLR2-, TLR7-, and TLR9-mediated cytokine production by peritoneal macrophages (48). It is also possible that signaling events downstream of Ly49Q regulate the TLR9 signaling cascade independent of the recruitment of phosphatases. This is evident from experiments showing increased tyrosine phosphorylation of proteins after mAb-mediated cross-linking of Ly49Q on macrophages (7).

Positive regulation of the TLR9 signaling cascade by Ly49Q upon stimulation with CpG-B ODN culminates in activation and translocation of IFN-I transcription factor to the nucleus. We show in this study that IFR7 translocation to the nucleus is impaired in Ly49Q-deficient pDC (Fig. 7B). Both IFR3 and IFR7 positively regulate IFN-I gene transcription (32, 33). Although IFR3 is constitutively expressed (32), IFR7 expression is induced by IFN-α and is required for the late-phase induction of IFN-α genes and feedback regulation of IFN-I production (34). However, these experiments were conducted in mouse embryonic fibroblast cells and may not be a true representation of IFR7 function and expression in pDC. pDC are unique in that they can secrete up to 1000 times more IFN-I than other cell type in response to microbial challenge (3). Furthermore, in pDC, TLR9-mediated IFN-I production is completely dependent on IFR7 (36). IFR7 is constitutively expressed at high levels in human pDC (46). Similarly, IFR7 protein can be detected at high levels in naive mouse pDC (Fig. 7A). Impaired translocation of IFR7 to the nucleus of Ly49Q-deficient pDC most likely has a significant impact on the expression of IFN-I genes and may explain the inability of these cells to produce IFN-α when stimulated with CpG-B ODN.

Recent work by Swiecki et al. (43) has demonstrated that IFN-α produced after viral infection of mice induces pDC death and significantly reduces their numbers. We recapitulate this phenomenon in the current study with CpG-B ODN challenge in both WT and Ly49Q-deficient mice (Fig. 3B). Because Ly49Q-deficient mice are impaired in the production of IFN-α through TLR9 activation by CpG-B ODN, one would expect lesser pDC death in these mice as compared with the WT mice. However, we observed similar levels of pDC death, measured by 7-AAD staining, in WT and Ly49Q-deficient mice receiving CpG-B ODN injections. IFN-α–induced pDC death, shown by Swiecki et al. (43), may require small levels of IFN-α. Although impaired, IFN-α production is not completely absent in Ly49Q-deficient mice. This small level of IFN-α upon CpG-B ODN challenge may be sufficient to induce pDC death in Ly49Q-deficient mice. Alternatively, Ly49Q-deficient pDC may be more susceptible to IFN-α–induced death or upon activation through mechanisms independent of IFN-α.

Our previous studies have demonstrated that Ly49Q plays an important role in membrane trafficking to regulate TLR9 signaling.
CpG-induced tubular endosomal extension is impaired in the absence of Ly49Q, resulting in altered distribution of TLR9 and CpG ODN in macrophages and pDC lacking Ly49Q (23). Consequently, cells lacking Ly49Q showed impaired cytokine production in response to CpG ODN (13). The role of Ly49Q and its ITIM as a sensor of microbial products has not been explored. Studies with human NK cells have implicated inhibitory KIR3DL2 in direct binding and trafficking of CpG ODN to early endosomes where TLR9 is encountered (47). Our preliminary experiments suggest a similar function of Ly49Q in CpG ODN uptake, which appears to be independent of Ly49Q ITIM (Fig. 8). Further experiments are required to address the role of Ly49Q and its ITIM in trafficking of CpG ODN to endosomal compartment and the nature of interaction with CpG ODN.

To take advantage of the discovery of MHC-I regulation of pDC function in mice, the existence of an analogous receptor performing the same function in human pDC needs to be confirmed. At present, the human functional analog of Ly49Q is unknown, although there are several promising candidates. CD300a/c are two related members of the Ig superfamily expressed on leukocytes, including NK cells and pDC. CD300a contains three ITIMs, whereas CD300c contains no obvious signaling motifs and most likely signals through an unidentified ITAM-containing adaptor. Ab cross-linking (cross-reactivity with both CD300a/c) results in enhanced IFN-α, but suppresses TNF-α and IL-6 production by pDC in response to CpG-ODN (49). The endogenous ligands are unknown, and it is not yet clear whether these effects are due to ITIM or ITAM signaling. Interestingly, human pDC preferentially express ILet (LILRA4), which couples with a signaling adapter to activate an ITAM-mediated signaling pathway. ILT7 protein directly binds to BST2 (CD317) protein, the expression of which is found on cells pre-exposed to IFN-α or on the surface of human cancer cells. Counterintuitively, the interaction between ILT7 and BST2 results in the reduction of IFN-α by pDC in response to TLR agonists (50). Finally, the C-type lectin-related dendritic cell immunoreceptor is expressed on pDC, contains a cytoplasmic ITIM, but inhibits IFN-I production upon Ab-mediated cross-linking (51). The physiological ligand of dendritic cell immunoreceptor is known. Although none of these human pDC receptors contain all obvious signaling motifs and most likely signals through an unidentified ITAM-containing adapter, Ab cross-linking (cross-reactivity with both CD300a/c) results in enhanced IFN-α, but suppresses TNF-α and IL-6 production by pDC in response to CpG-ODN (49). The endogenous ligands are unknown, and it is not yet clear whether these effects are due to ITIM or ITAM signaling. Interestingly, human pDC preferentially express ILet (LILRA4), which couples with a signaling adapter to activate an ITAM-mediated signaling pathway. ILT7 protein directly binds to BST2 (CD317) protein, the expression of which is found on cells pre-exposed to IFN-α or on the surface of human cancer cells. Counterintuitively, the interaction between ILT7 and BST2 results in the reduction of IFN-α by pDC in response to TLR agonists (50). Finally, the C-type lectin-related dendritic cell immunoreceptor is expressed on pDC, contains a cytoplasmic ITIM, but inhibits IFN-I production upon Ab-mediated cross-linking (51). The physiological ligand of dendritic cell immunoreceptor is unknown. Although none of these human pDC receptors contain all obvious signaling motifs and most likely signals through an unidentified ITAM-containing adapter, Ab cross-linking (cross-reactivity with both CD300a/c) results in enhanced IFN-α, but suppresses TNF-α and IL-6 production by pDC in response to CpG-ODN (49). The endogenous ligands are unknown, and it is not yet clear whether these effects are due to ITIM or ITAM signaling. Interestingly, human pDC preferentially express ILet (LILRA4), which couples with a signaling adapter to activate an ITAM-mediated signaling pathway. ILT7 protein directly binds to BST2 (CD317) protein, the expression of which is found on cells pre-exposed to IFN-α or on the surface of human cancer cells. Counterintuitively, the interaction between ILT7 and BST2 results in the reduction of IFN-α by pDC in response to TLR agonists (50). Finally, the C-type lectin-related dendritic cell immunoreceptor is expressed on pDC, contains a cytoplasmic ITIM, but inhibits IFN-I production upon Ab-mediated cross-linking (51).

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Disclosures

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