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Development of Murine Plasmacytoid Dendritic Cells Defined by Increased Expression of an Inhibitory NK Receptor, Ly49Q[1]

Yoshiki Omatsu,* Tomonori Iyoda,* Yukino Kimura,* Akiko Maki,† Masaki Ishimori,* Noriko Toyama-Sorimachi,† and Kayo Inaba[2]*

Plasmacytoid dendritic cells (PDCs) are defined in mice by a unique combination of markers: CD11c, B220, and Ly6C/G. We have reported previously that PDCs express Ly49Q, a lectin-type killer cell inhibitory receptor. We now find that different expression levels of Ly49Q define sequential developmental stages of PDCs in bone marrow. Although PDCs in spleen and lymph nodes express high levels of Ly49Q, a significant portion of $CD11c^+B220^+$ PDCs in bone marrow lack Ly49Q, as well as the CD4 and MHC II. Purified $Ly49Q^-$ marrow PDCs spontaneously up-regulate Ly49Q after overnight culture without cell proliferation and acquire most features of typical PDCs in spleen. When exposed to TLR ligands, such as CpG-oligodeoxynucleotide and hemagglutinating virus of Japan (Sendai virus), $Ly49Q^-$ PDCs increase CD86 and MHC class II expression but produce less IFN-$\alpha\beta$, IL-6, and IL-12p70 than $Ly49Q^+$ PDCs, although they are able to produce comparable amounts of TNF-α. However, interestingly, $Ly49Q^-$ PDCs do not produce TNF-α in response to the TLR2 ligand, $Pam3SCK_4$, whereas $Ly49Q^+$ PDCs did. Therefore, Ly49Q is a new marker to identify a precursor form of PDCs that participates in innate immunity. *The Journal of Immunology,* **2005, 174: 6657–6662.**

Dendritic cells (DCs)[3] are comprised of a number of subsets with distinct functions. An example is plasmacytoid DCs (PDCs), also known as type I IFN-producing cells because of their very high levels of IFN production during encounter with viruses or select TLR ligands (1–5). These stimuli also activate PDCs to produce TNF-α, IL-6, and IL-12p70 (6–10). With certain viruses, especially HSV (11) and CMV (12–14), PDCs bridge innate to adaptive immunity.

Murine PDCs are $CD11c^{int}CD11b^-B220^+Ly6C^+Gr\text{-}1^+$ cells (6, 15). Recently, murine PDCs have been identified with a new mAb 120G8 (16). The 120G8 mAb displayed a high specificity against both activated and resting PDCs, reacting with most of the PDC population in various tissues. Other studies have demonstrated some heterogeneity in PDCs, involving CD4 and CD8 expression and turnover rate (17). Although it is known that proliferating progenitors exist in the bone marrow (18–21), little attention has been paid to intermediate stages of PDC development.

Ly49Q, an ITIM-bearing inhibitory receptor, is a member of the Ly49 family of murine NK receptors, but it is unusual in several respects (22). First, it is not expressed on NK or NKT cells but instead on $Gr\text{-}1^+$ myeloid lineage cells. Second, the expression on macrophages is regulated during development and activation and is enhanced greatly by IFN-γ. Third, signals via Ly49Q rapidly induce morphological changes in macrophages, including formation of polarized actin structures with filopodia-like structure on one side and lamellipodia-like structure on the other side. In addition, we recently found that Ly49Q is expressed on PDCs in the spleen and a portion of cultured DCs generated from bone marrow using cytokines such as Flt3 ligand and GM-CSF.[4]

In the present study, we will show that sequential maturational stages of PDCs can be identified in the marrow on the basis of expression levels of Ly49Q. PDCs lacking Ly49Q are immature in terms of lower production of cytokines, including IFN-$\alpha\beta$, IL-6, and IL-12p70, upon TLR-dependent stimulation compared with $Ly49Q^+$ PDCs, but $Ly49Q^-$ PDCs can produce TNF-α. Importantly, these cells differentiate to typical PDCs by up-regulating Ly49Q and MHC class II. These findings provide evidence that marrow contains an early developmental stage of PDC, with a distinct cytokine profile.

Materials and Methods

Mice

Both male and female BALB/c mice were purchased from Japan SLC or Nippon Clea and maintained under specific pathogen-free conditions and

*Department of Animal Development and Physiology, Graduate School of Biostudies, Kyoto University, Kyoto, Japan; and †Department of Gastroenterology, Research Institute, International Medical Center of Japan, Toyama, Tokyo, Japan

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[2] Address correspondence and reprint requests to Dr. Kayo Inaba, Department of Animal Development and Physiology, Graduate School of Biostudies, Kyoto University, Kitashiarakawa-Oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan. E-mail address: kayo@lif.kyoto-u.ac.jp; or 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; PDC, plasmacytoid DC; SA, streptavidin; ODN, oligodeoxynucleotide; HVJ, hemagglutinating virus of Japan (Sendai virus); HAU, hemagglutinating unit; SAC, *Staphylococcus aureus*.

[4] N. Toyama-Sorimachi, Y. Omatsu, A. Onoda, Y. Tsujimura, T. Iyoda, A. Maki, H. Sorimachi, T. Dohi, K. Inaba, and H. Karasuyama. Inhibitory NK receptor Ly49Q is expressed on subsets of dendritic cells in a cellular maturation- and cytokine stimulation-dependent manner. *Submitted for publication.*

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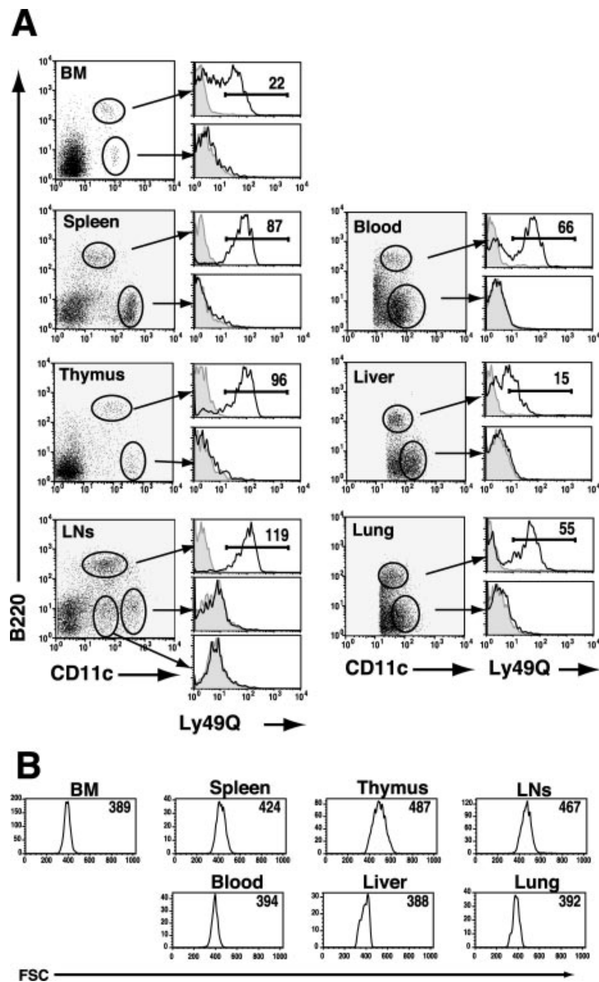

FIGURE 1. Expression of Ly49Q on PDCs in lymphoid and nonlymphoid organs. Low-density cells prepared from indicated tissues were stained PE-CD19, PE-CD3, FITC-B220, and APC-CD11c mAbs with biotinylated Ly49Q (open histograms) or control rat IgG2a Ab (shaded histograms) and SA-PerCP-Cy5.5. Cells corresponding to CD11c$^+$B220$^+$ PDCs and CD11c$^+$ B220$^-$ conventional DCs were analyzed separately for expression of Ly49Q (*A*). Horizontal bars show gate setting for cell size analysis of Ly49Q$^+$ PDCs (*B*). Numbers in histograms in histogram of *A* indicate the mean MFI values of MHC class II expressed by Ly49Q$^+$ PDCs. BM, bone marrow; LNs, lymph nodes; FSC, forward light scatter.

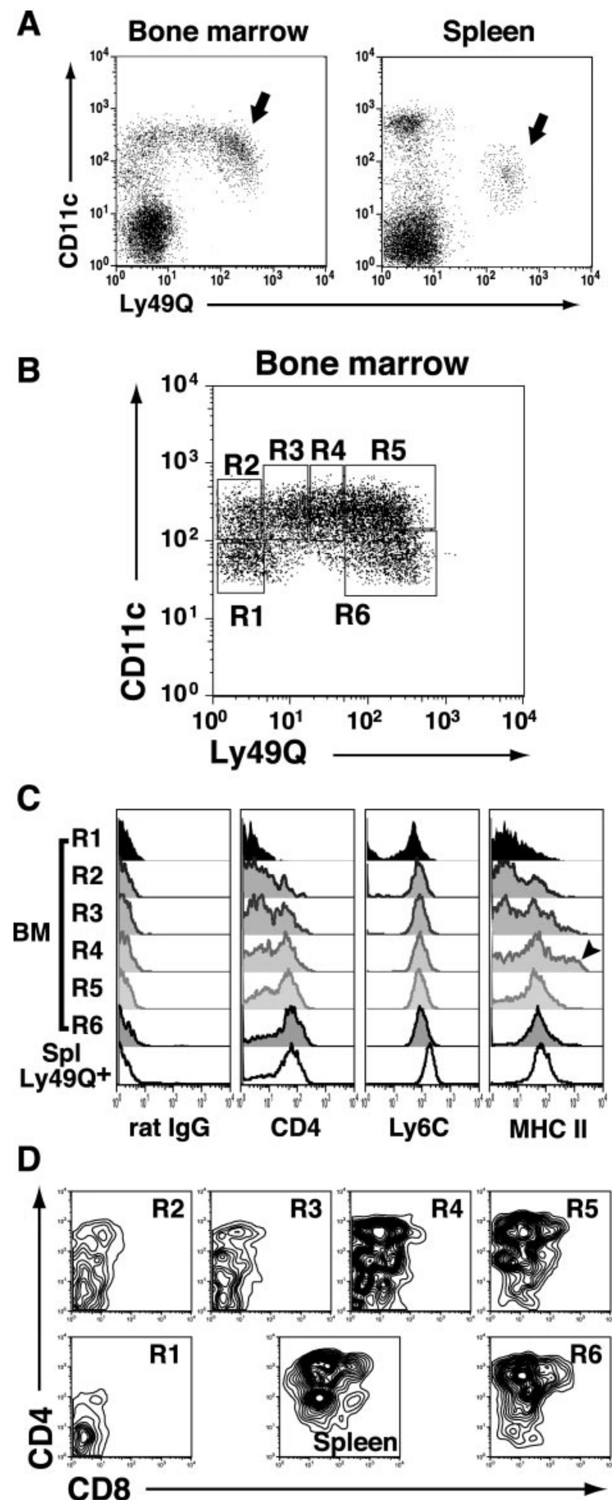

FIGURE 2. Comparison of PDC phenotype in bone marrow and spleen. After staining low-density bone marrow and spleen cells with PE-conjugated CD19, CD3 and DX5, FITC-B220, and APC-CD11c mAbs with biotinylated Ly49Q (open histograms) or control rat IgG2a Ab (shaded histograms) and SA-PerCP-Cy5.5 (*A*), putative CD11c$^+$B220$^+$ PDCs were divided into six subpopulations depending on expression levels of CD11c and Ly49Q (*B*). For phenotyping, bone marrow cells were first depleted of CD19, CD3, and DX5-positive cells with magnetic beads and stained with various Abs (*C* and *D*).

used at 8–10 wk of age. The research was performed according to institutional Guidelines for Animal Use and Experimentation.

Abs and reagents

All fluorescence dye-conjugated mAbs and streptavidins (SAs) used were purchased from BD Pharmingen or from eBioscience. Purified and biotinylated-Ly49Q mAb were described previously (22). Biotinylated Ly6C (ER-MP20) was obtained from BMA Biomedicals. All other biotinylated mAbs were from BD Pharmingen or from eBioscience. Rat mAbs for MHC class II (TIB120, M5/114.15.2), CD205 (HB290, DEC-205), and CD16/32 (HB-197: 2.4G2) were a gift from R. M. Steinman (Rockefeller University, New York, NY). Ab-conjugated microbeads were purchased from Miltenyi Biotec, and SA-BD IMag was purchased from BD Pharmingen. Pam3Cys-Ser-(Lys)4 (Pam3CSK$_4$) was purchased from Calbiochem-Novabiochem). CpG-ODN1826 (5′-TCCATGACGTTCCTCACGTT-3′), CpG-ODN1585 (5′-GGGGTCAACGTTGAGGGGGG-3′), and control oligodeoxynucleotide (ODN) (5′-TCCATGAGCTTCCTGATGCT-3′) were obtained from Hokkaido System Science.

Cell preparations and cultures

Collagenase-digested, low-density cells from spleens, lymph nodes, and thymi were prepared as described previously (23). CD11c$^+$ DC fractions were positively selected using AutoMACS (Miltenyi Biotec). In some experiments, T, B, and NK cells were depleted by microbeads using anti-CD3, anti-CD19, CD11b, and DX5 mAbs, and additional B220$^+$ cells were

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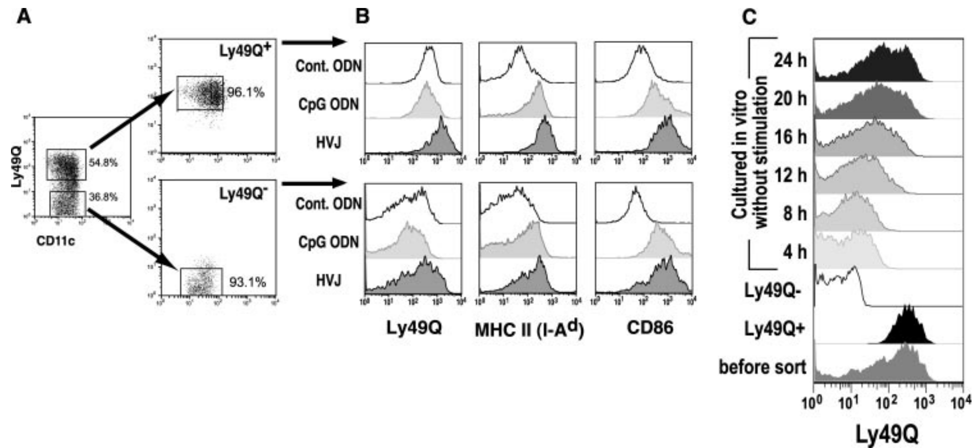

FIGURE 3. Transition of Ly49Q$^-$ PDCs to Ly49Q$^+$ PDCs. Cells were stained with the combination of mAbs as described in Fig. 1*C*, and subsequently, Ly49Q$^+$ and Ly49Q$^-$ PDCs were purified from cells gated on CD11c$^+$B220$^+$ by cell sorter (*A*). Then, they were cultured with control-ODN, CpG-ODN (3 μM), or HVJ (10 HAU/ml) for 18–20 h (*B*) or without any antigenic stimulus for indicated periods (*C*). The cells were harvested and stained again with anti-Ly49Q mAb in combination with anti-I-A^d, anti-CD86, and isotype-matched control Abs. Histograms display staining patterns of Ly49Q$^+$ (*upper panels*) and Ly49Q$^-$ (*lower panels*) cells (*B*). Stimulation with CpG-ODN and HVJ resulted in the increased expression of MHC class II and CD86 in both Ly49Q$^+$ and Ly49Q$^-$ PDCs. Although augmentation of I-A^d and CD86 expression was not observed with control-ODN, increased expression of Ly49Q in Ly49Q$^-$ PDCs was detected at significant levels (*B*). Such spontaneous increases in Ly49Q expression was also detected on Ly49Q$^-$ PDCs as early as 4 h of incubation, and the expression levels rose gradually thereafter (*C*).

enriched. For liver and lung, leukocytes were prepared from perfused tissues as described previously (23). For isolation of PDC subpopulations, bone marrow CD11c$^+$ cells were enriched positively or negatively using microbeads and subsequently stained with anti-CD11c and anti-B220 mAbs in combination with anti-Ly49Q mAb. The stained cells were separated by FACSVantage (BD Biosciences). Isolated PDCs were cultured in RPMI 1640 medium containing 10% FCS at 7.5 × 10^4/well in the absence or presence of control ODN, CpG-ODN (each 3 μM), hemagglutinating virus of Japan (Sendai virus) (HVJ) (3–10 hemagglutinating units (HAU)/ml), and Pam3CSK$_4$ (1 μg/ml) for 24 h.

Flow cytometric analyses

Bulk or low-density leukocytes prepared from various lymphoid organs were stained with PE-CD19, PE-CD3, FITC-B220, and APC-CD11c mAbs in combination with biotinylated Ly49Q and SA-PerCP-Cy5.5. Before staining, cells were incubated with 2.4G2 mAb to prevent nonspecific mAb binding. Cells stained were acquired by FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star). Results shown are a representative of at least five separate experiments.

Quantitation of cytokine production

Purified Ly49Q$^+$ and Ly49Q$^-$ PDCs (7.5 × 10^4 cells/well) were incubated with CpG-ODN or control ODN (3 μM). In some experiments, PDCs were infected with HVJ (10 HAU/ml). After 18 h of incubation, secreted cytokines were measured using BD Cytometric Bead Array (BD Pharmingen). Production of IFN-$\alpha\beta$ was measured by bioassay using L929 cells as described previously (24). For intracellular IFN-α staining, enriched CD11c$^+$ bone marrow cells were cultured at 10^6 cells/well in 96-well round-bottom plates in the presence of CpG-ODN (1826) at 1 μM for 2 h followed by addition of 10 μg/ml brefeldin A and further cultured for 3h. After washing, cells were incubated with 2.4G2 mAb and subsequently stained with PE-CD11c, APC-B220, and biotinylated anti-Ly49Q plus SA-PerCP-Cy5.5. Then, cytoplasmic stainings were performed using anti-IFN-α (PBL Biochemical Laboratories) and FITC-anti-rat IgG1 as described previously (25). Results shown are a representative of three separate experiments.

Statistical analyses

Data are expressed as the mean values and SD of triplicate experiments. Statistical significances were determined by the Student *t* test. Differences were considered to be significant for p values <0.5.

Results

Heterogeneous expression of Ly49Q in marrow and nonlymphoid tissues

We previously detected the expression of Ly49Q on PDCs developing from bone marrow progenitors with Flt3L in vitro.[4] Because the expression of surface molecules on DCs varies with their tissue localization and maturational state (17, 26, 27), we studied Ly49Q expression on PDCs and conventional DCs from various lymphoid and nonlymphoid organs of BALB/c mice. In each, we identified CD11c^{high} B220$^-$ conventional DCs as well as CD11c^{int}B220$^+$ PDCs. As shown in Fig. 1*A*, PDCs in all organs examined expressed Ly49Q. In contrast, the conventional DCs failed to express Ly49Q. The expression levels of Ly49Q as well as MHC class II (the mean fluorescence intensity values are indicated in histograms) on PDCs differed considerably in the tissues we studied (Fig. 1*A*). Interestingly, Ly49Q$^+$ PDCs in the thymus and secondary lymphoid organs were larger (higher forward scatter) in size than those in lung, liver, blood, and marrow (mean forward light scatter signal is shown in each panel of Fig. 1*B*). Ly49Q was highly expressed on most PDCs in s.c. lymph nodes as well as spleen, whereas in thymus, bone marrow, blood, lung, and liver, a significant portion of PDCs was negative or low for Ly49Q. Especially in bone marrow, the frequency of Ly49Q$^-$ PDCs was high (Fig. 1*A*, *top left*).

Regarding Ly49Q$^-$ cells in the CD11c$^+$B220$^+$ PDC populations, there was the possibility that cell types other than PDCs could be expressing CD11c and B220, especially NK cells. NK1.1$^+$CD3$^-$ NK cells in C57BL/6 mice and DX5$^+$ NK cells in BALB/c mice did express low levels of CD11c and B220, whereas NK1.1$^+$CD3$^+$ NKT cells did not. However, the NK cells lacked Gr-1 and Ly6C and were smaller in cell size than PDCs (data not shown). In contrast, the Gr-1$^+$ Ly49Q$^-$ cells in the B220$^+$CD11c$^+$ population from bone marrow (i.e., presumptive PDCs) had high levels of Ly6C and lacked the NK marker DX5 (data not shown). These results indicate that there is a Ly49Q$^-$ fraction of CD11c$^+$B220$^+$ presumptive PDCs in many tissues, although it is rare in organized peripheral lymphoid tissue such as the spleen and lymph nodes.

Initial evidence for PDC differentiation according to Ly49Q expression in the bone marrow

Because Ly49Q$^-$ cells were so abundant in the marrow, we looked more carefully at the levels of CD11c and Ly49Q as well as other surface molecules expressed on DCs. When bone marrow and splenic B220$^+$ PDCs were compared for the expression of CD11c

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and Ly49Q, splenic PDCs were Ly49Q$^+$ with intermediate expression of CD11c as previously reported (15, 26), whereas PDCs in bone marrow were high in CD11c expression relative to those in spleen and heterogeneous in Ly49Q expression (Fig. 2*A*). To further characterize PDCs in the marrow, CD11c$^+$B220$^+$ cells were enriched by depleting CD19$^+$ B, CD3$^+$ T, and DX5$^+$ NK cells and analyzed for the expression of various markers. As shown in Fig. 2*B*, we arbitrarily divided the cells into six populations based on the expression levels of CD11c and Ly49Q. We could tentatively arrange these subsets into a PDC developmental pathway, using the other PDC markers indicated (Fig. 2*B*). Specifically, as the subsets expressed more Ly49Q (moving from R1 to R6), they also acquired increased levels of CD4 and MHC II (Fig. 2*C*). Likewise, if we double labeled these subsets for CD4 and CD8, we found progressive up-regulation of both markers concomitant with increasing Ly49Q expression (Fig. 2*D*), eventually resembling the PDCs in spleen (17). However, curiously, some of PDCs that expressed low and intermediate level of Ly49Q were high in MHC class II (arrowheads in Fig. 2*C*), although there was no difference in the expression of MHC class I (data not shown). We hypothesized from these data that bone marrow contains PDCs in different stages of differentiation, with the Ly49Q$^-$ but CD11c$^+$B220$^+$Ly6C$^+$ cells representing PDC precursors at earlier developmental stage.

Ly49Q$^-$ PDCs differentiate to express Ly49Q

To assess this hypothesis, we cultured the purified Ly49Q$^-$ and Ly49Q$^+$ marrow PDCs (Fig. 3*A*) by gating on R1-3 and R5-6, according to the arbitrary classification in Fig. 2*B*. The Ly49Q$^-$ cells spontaneously and gradually increased Ly49Q expression following overnight culture in medium without any cell proliferation (Fig. 3*C*). When we added CpG-ODN or HVJ virus, these TLR ligands led to increased expression of MHC II and CD86 in both Ly49Q$^-$ and Ly49Q$^+$ PDCs, although Ly49Q$^-$ PDC responded more vigorously than Ly49Q$^+$ PDCs. Only the virus was able to induce enhanced expression of Ly49Q in the initial Ly49Q$^-$ population above the medium control (Fig. 3*B*). Morphologically, the CpG-ODN-stimulated cells became larger and irregular in shape in 18-h culture (data not shown). These findings strengthen the notion that Ly49Q$^-$ marrow PDCs can be progenitors of more differentiated PDCs found in lymphoid tissues.

Differences in cytokine producing abilities between Ly49Q$^-$ and Ly49Q$^+$ PDCs

We next stimulated Ly49Q$^-$ and Ly49Q$^+$ PDCs with CpG-ODN and HVJ to assess cytokine production. Ly49Q$^+$ PDCs produced about twice the level of IFN-$\alpha\beta$ with either stimulus (Fig. 4, *A* and *B*). Intracellular staining for IFN-α confirmed that Ly49Q$^+$ PDCs were much better at producing IFN-α in response to CpG-ODN than Ly49Q$^-$ PDCs (Fig. 4*C*). As in the case of IFN-$\alpha\beta$, higher amounts of IL-12p70 and IL-6 were produced from Ly49Q$^+$ PDCs than Ly49Q$^-$ PDCs, but TNF-α production was nearly comparable between them (Fig. 4, *A* and *B*). Interestingly, when Ly49Q$^+$ PDC and Ly49Q$^-$ PDC populations were stimulated with Pam3CSK$_4$, a TLR2 ligand, Ly49Q$^+$ marrow PDCs produced much larger amounts of TNF-α than Ly49Q$^-$ PDCs (Fig. 5*A*). As reported by O'Keeffe et al. (10) using *Staphylococcus aureus* (SAC), the amount of TNF-α produced by CD4$^-$ spleen PDCs was higher than CD4$^-$ spleen PDCs. However, all PDC populations from bone marrow and spleen produced no IL-6, IL-12p70, and IFN-$\alpha\beta$ in response to Pam3CSK$_4$ (data not shown), although Ly49Q$^+$ and Ly49Q$^-$ PDCs augmented expression of CD86 and MHC class II (Fig. 5*B*). The increase in CD86 and MHC II were again greater in Ly49Q$^+$ than Ly49Q$^-$ populations, as was the case with CpG-ODN and HVJ.

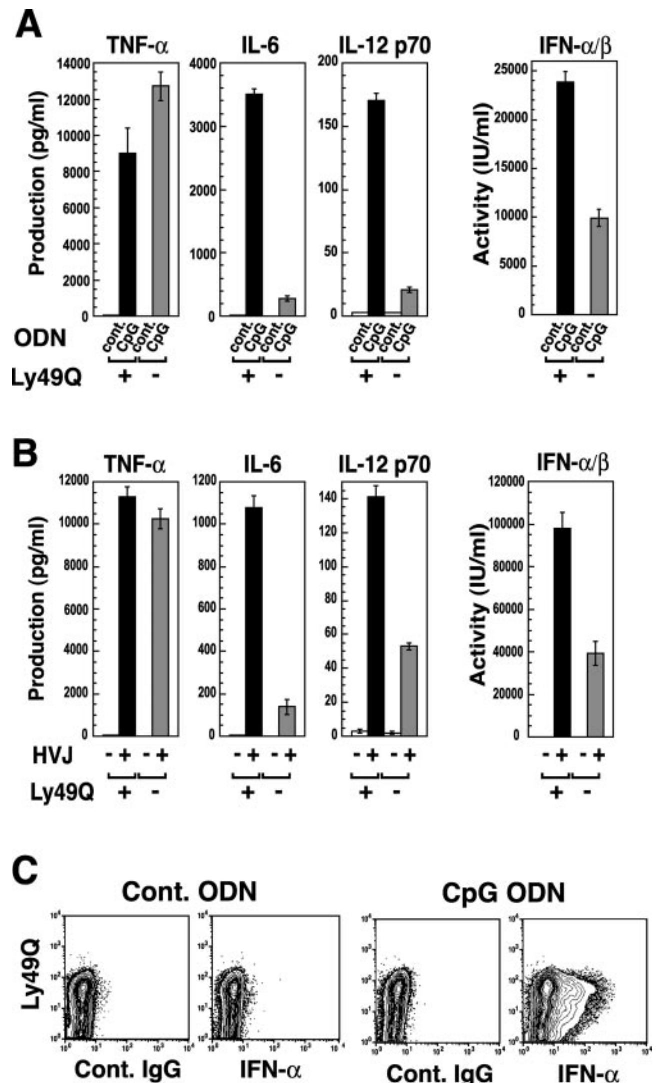

FIGURE 4. Cytokine-producing abilities of Ly49Q$^+$ and Ly49Q$^-$ PDCs. Purified Ly49Q$^+$ and Ly49Q$^-$ PDCs were cultured with control ODN, CpG-ODN (*A*), or HVJ (*B*) for 18–20 h. Then, cytokine activities secreted into culture supernatant were examined with a cytometric bead array method for TNF-α, IL-12 p70, and IL-6 and by bioassays for IFN-$\alpha\beta$. IFN-α production was also assessed by intracellular staining of cells cultured in the presence of 1 μM CpG-ODN (1826) for 5 h (*C*).

Discussion

In the present study, we have shown that Ly49Q is a useful marker for the development of PDCs in situ, especially in bone marrow. In contrast to peripheral lymphoid organs, CD11c$^+$B220$^+$ PDCs in marrow were heterogeneous in Ly49Q expression. The Ly49Q$^-$ and Ly49Q^{low} PDC populations were mostly CD4$^-$ cells. However, these cells successively and gradually increased Ly49Q expression after overnight culture in the absence of any stimulation such as TLR ligands, and this process took place without cell proliferation. Arbitrarily gated Ly49Q^{high} PDCs in marrow eventually expressed nearly comparable levels of CD4 and MHC II with splenic PDCs. The expression levels of Ly49Q were well correlated with the expression of CD4 as well as MHC class II. Therefore, Ly49Q$^-$ PDCs in marrow are possibly less differentiated PDCs. Furthermore, the increase in CD4 expression appeared to take place before that of CD8, suggesting that CD4$^+$CD8$^+$ PDCs were derived from CD4$^+$ PDCs.

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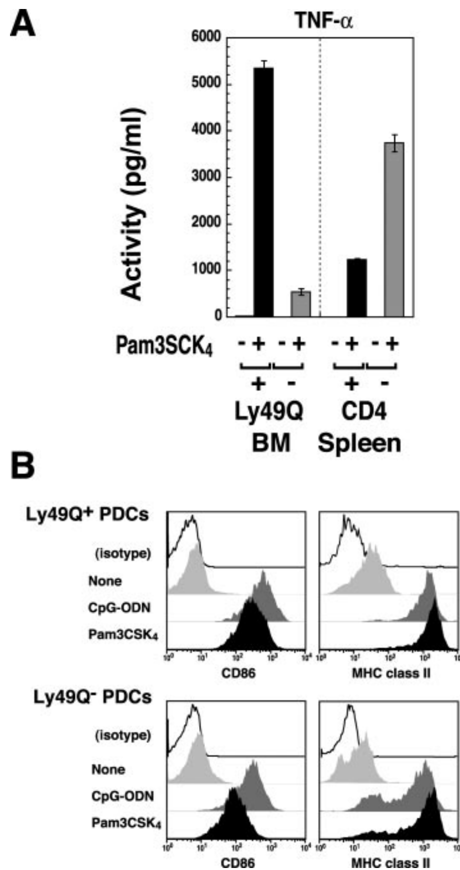

FIGURE 5. Distinctive responsiveness of Ly49Q$^+$ and Ly49Q$^-$ PDCs to Pam3CSK$_4$. Purified Ly49Q$^+$ and Ly49Q$^-$ cells were cultured in the presence of 1 μg/ml Pam3CSK$_4$ for 18–20 h, and cytokine production (*A*) and up-regulation of MHC class II and CD86 (*B*) were examined.

Regarding function, Ly49Q$^-$ PDCs produced lower amounts of IL-6, IL-12p70, and IFN-$\alpha\beta$ relative to Ly49Q$^+$ PDCs in response to CpG-ODN as well as HVJ. There have been reports that cord blood PDCs show reduced IFN-α production with CpG (28) and that newly developed CD11c^{high}CD45RA$^+$B220$^+$ splenic PDCs (using Flt3 ligand and newborn mice), most of which are negative for CD4, are less able to produce IFN-α and IL-12 in response to HSV-1 than PDCs from normal adult mice (29). Ly49Q$^-$ marrow PDCs are possibly related in differentiation stage with the newly developed CD11c^{high}CD45RA$^+$B220$^+$ splenic PDCs induced by Flt3 ligand, because PDCs in marrow also expressed high levels of CD11c relative to spleen PDCs.

It is of note that a small but significant portion of Ly49Q$^-$ PDCs was identified not only in marrow but also in blood, thymus, and nonlymphoid organs, including liver and lung. This heterogeneity was also evident in the fact that the expression of MHC class II and cell size of PDCs differs by organs.

A significant feature of Ly49Q$^+$ PDCs in marrow is their responsiveness to Pam3CSK$_4$, a synthetic TLR2 ligand (30), as manifest by production of TNF-α, but not other cytokines, especially IFN-$\alpha\beta$. Spleen CD4$^-$ PDCs also responded to Pam3CSK$_4$ better than CD4$^+$ PDCs, as in the case of IFN-α production to SAC (17). Although Ly49Q$^-$ PDCs are comprised of CD4$^-$ cells, they are the least competent to respond to Pam3CSK$_4$. Taken together with the observation that marrow Ly49Q$^+$ PDCs, which are heterogeneous in CD4 and CD11c expression, are the most potent responder, the responsiveness to TLR2 ligand may primarily occur at a transitional stage from CD4$^-$ to CD4$^+$ PDCs.

TLR2 has been documented to be expressed predominantly on monocytes and CD11c$^+$ DCs but not on CD11c$^-$ PDCs in human peripheral blood (31, 32). However, CD11c$^-$ PDCs in blood and lesional skin of patients with erythema migrans increase their expression of TLR1, TLR2, and TLR4 (33). In addition, murine thymic PDCs are also reported to express low but significant levels of TLR2, TLR3, and TLR4 (34). We also confirmed in our preliminary experiment the expression of TLR2 on PDCs by FACS (data not shown).

Pam3CSK$_4$ did not stimulate to produce IFN-$\alpha\beta$ from any PDC populations. Cell wall components of SAC are recognized selectively by TLR2 (35, 36), but TLR2-deficient macrophages are able to produce reduced but significant levels of cytokines in response to SAC (37). Therefore, other components of SAC as well as different sets of TLRs may contribute to induce IFN-α productions by PDCs.

During preparation of this article, Kamogawa-Schifter et al. (38) have also reported that BM PDCs can be divided into two subsets. In conclusion, we have identified developmental stages of PDCs from mouse bone marrow by their different expression levels of an inhibitory NK receptor, Ly49Q.

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Disclosures

The authors have no financial conflict of interest.

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