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The Roles of Toll-Like Receptor 9, MyD88, and DNA-Dependent Protein Kinase Catalytic Subunit in the Effects of Two Distinct CpG DNAs on Dendritic Cell Subsets[1]

Hiroaki Hemmi,*† Tsuneyasu Kaisho,*†‡ Kiyoshi Takeda,*† and Shizuo Akira[2]*†

Oligodeoxynucleotides containing unmethylated CpG motifs (CpG DNAs) can function as powerful immune adjuvants by activating APC. Compared with conventional phosphorothioate-backbone CpG DNAs, another type of CpG DNAs, called an A or D type (A/D-type), possesses higher ability to induce IFN-α production. Conventional CpG DNAs can exert their activity through Toll-like receptor 9 (TLR9) signaling, which depends on a cytoplasmic adapter, MyD88. However, it remains unknown how A/D-type CpG DNAs exhibit their immunostimulatory function. In this study we have investigated murine dendritic cell (DC) responses to these two distinct CpG DNAs. Not only splenic, but also in vitro bone marrow-derived, DCs could produce larger amounts of IFN-α in response to A/D-type CpG DNAs compared with conventional CpG DNAs. This IFN-α production was mainly due to the B220$^+$ DC subset. On the other hand, the B220$^-$ DC subset responded similarly to both CpG DNAs in terms of costimulatory molecule up-regulation and IL-12 induction. IFN-α, but not IL-12, induction was dependent on type I IFN. However, all activities of both CpG DNAs were abolished in TLR9- and MyD88-, but were retained in DNA-PKcs-deficient DCs. This study demonstrates that the TLR9-MyD88 signaling pathway is essential for all DC responses to both types of CpG DNAs. ***The Journal of Immunology*****, 2003, 170: 3059–3064.**

Bacterial DNA can stimulate innate immunity in mammals (1–3). This immunostimulatory activity depends on the unmethylated CpG motif, which is abundantly present in microbes. Synthetic oligodeoxynucleotides containing the unmethylated CpG motif (CpG DNAs) are equivalent to bacterial DNA in the immunostimulatory activity. CpG DNAs can induce splenic B cell proliferation, dendritic cell (DC)[3] maturation, and cytokine production from a variety of immune cells (1–3). These CpG DNAs are phosphorothioate-modified oligodeoxynucleotides called K-type CpG DNAs or CpG-B (conventional CpG DNAs). Through the screening of a variety of CpG DNAs, another type of CpG DNAs with a distinct function was identified (4–7). These are termed D-type CpG DNAs or CpG-A (A/D-type CpG DNAs) and are structurally different from conventional CpG DNAs because they carry a phosphorothioate-modified polyguanosine (polyG) stretch at the 5′ and 3′ ends and a phosphodiester backbone CpG motif at the central position. The function of A/D-type CpG DNAs has been extensively characterized in the human system (4, 5). A/D-type CpG DNAs can induce cytokine production in a variety of cells, but exhibit weaker ability to induce proliferation and IgM production of splenocytes than conventional CpG DNAs (4). Notably, A/D-type CpG DNAs have greater ability to induce IFN-α production from plasmacytoid DC (PDC) and IFN-γ from NK cells (5, 6).

We previously demonstrated that Toll-like receptor 9 (TLR9) is essential for CpG DNA-induced immune responses based on the fact that TLR9-deficient (TLR9$^{-/-}$) mice are refractory to CpG DNAs (8). In addition, TLR9 expression is sufficient to confer responsiveness to CpG DNA on a human kidney cell line (9, 10). Some CpG DNAs can activate human immune cells more efficiently than murine ones, while others can activate murine cells more effectively than human ones. This species-specific response is reconstituted by the expression of human or murine TLR9 on an otherwise refractory cell line, suggesting that TLR9 is also critical for the species-specific function of CpG DNAs (9). However, because all these experiments were performed with conventional CpG DNAs, the cellular and molecular mechanisms of how immune cells are activated by A/D-type CpG DNAs remain unelucidated.

In this study we have investigated how these CpG DNAs activate DCs, which play crucial roles in host defense by linking innate and adaptive immunities. Both types of CpG DNAs could induce IL-12 secretion from DCs. However, compared with conventional CpG DNAs, A/D-type CpG DNAs could induce greater amounts of IFN-α production from DCs. A B220$^+$ DC subset, which is considered to be a murine counterpart of human PDC, was mainly responsible for IFN-α production induced by CpG DNAs. Both conventional and A/D-type CpG DNAs required the TLR9 signaling system and could induce IFN regulatory factor 7 (IRF7) mRNA up-regulation at similar levels. Thus, common signaling pathways are involved in the effects of distinct types of CpG DNAs on murine DC subsets.

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[3] Abbreviations used in this paper: DC, dendritic cell; BM, bone marrow; CpG DNA, oligodeoxynucleotides containing the unmethylated CpG motif; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; Flt3L, Flt3 ligand; IRF7, IFN regulatory factor 7; PDC, plasmacytoid DC; polyG, polyguanosine; SR-A, scavenger receptor A; TLR, Toll-like receptor.

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Materials and Methods

Mice

C57BL/6J mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). MyD88-deficient (MyD88$^{-/-}$) mice were established as described previously (11) and backcrossed over eight times with C57BL/6 mice. TLR9$^{-/-}$ mice were generated as described previously (8). DNA-dependent protein kinase catalytic subunit-deficient (DNA-PKcs$^{-/-}$) mice were provided by Dr. F. W. Alt (Harvard Medical School, Boston, MA) (12). IFN-α/βR-deficient (IFN-α/βR$^{-/-}$) mice were purchased from B&K Universal Ltd. (Hull, U.K.).

Reagents and Abs

Synthesized oligodeoxynucleotides were purchased from Hokkaido System Science (Sapporo, Japan). The sequences and backbones of oligodeoxynucleotides are: ODN1668, tccatgacgttcctgatgct (13); D19, ggTGC ATCGATGCAgggggG (4); and control D, ggTGCATGCATGCAgggggG (4). Capital and lowercase letters in parentheses indicate bases with phosphodiester and phosphorothioate-modified backbones, respectively. Abs against mouse CD11c (clone HL3), CD40 (clone 3/23), CD86 (clone GL1), and B220 (clone RA3-6B2) were purchased from BD PharMingen (San Diego, CA)

Preparation of DCs

To prepare splenocytes containing DCs, spleens were cut unto small fragments and incubated with RPMI 1640 medium containing 400 U/ml collagenase (Wako Pure Chemical Industries, Osaka, Japan) and 15 μg/ml DNase (Sigma-Aldrich, St. Louis, MO) at 37°C for 20 min. For the last 5 min, EDTA was added at 5 mM. Single-cell suspensions were prepared after RBC lysis. CD11c$^+$ cells were purified by MACS with anti-CD11c microbeads and used as splenic DCs. Enriched cells contained >90% CD11c$^+$ cells.

To prepare in vitro bone marrow-derived DCs (BMDCs), BM cells were prepared from femora and tibia and passed through nylon mesh. Then cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 μM 2-ME, and 100 ng/ml human Flt3 ligand (Flt3L; PeproTech EC, London, U.K.). After 6–8 days, the cells were used as Flt3L-induced BMDCs (Flt3L-BMDCs) for additional experiments. Flt3L-BMDCs on days 6–8 contained 80–90% CD11c$^+$ cells, as described previously (14).

Flow cytometry

Splenic CD11c$^+$DCs or Flt3L-BMDCs were first incubated with biotinylated anti-CD11c and then with PE-conjugated anti-B220 and CyChrome-conjugated streptavidin. As indicated in Fig. 2, B220$^+$ and B220$^-$ cells were sorted using a FACSVantage (BD Bioscience, Mountain View, CA).

To evaluate surface expression levels of costimulatory molecules, cells were stained with biotinylated anti-CD40 and FITC-labeled anti-CD86, developed with PE-conjugated streptavidin, and analyzed on a FACSCalibur (BD Bioscience).

Measurement of cytokine production

Cells were seeded into 96-well plates at 2×10^6 cells/ml, or as otherwise indicated, in RPMI 1640 with 10% FCS and stimulated with various doses of synthesized oligodeoxynucleotides for 24 h. Culture supernatants were collected and analyzed for cytokine production. Cytokine concentrations in the supernatants were measured with ELISA. ELISA kits for mouse IFN-α were purchased from PBL Biomedical Laboratories (New Brunswick, NJ). ELISA kits for mouse TNF-α and IL-12 p40 were obtained from TECHNE Corp. (Minneapolis, MN).

Northern blot analysis

Total RNAs were isolated using Sepazol-RNA I (Nacalai Tesque, Kyoto, Japan), electrophoresed, and transferred to nylon membranes. Hybridization was performed with the indicated cDNA probes as described previously (11). cDNA probes specific for IFN-β, IL-12 p40, and IRF7 were obtained though the previously published subtractive screenings (15). The IFN-α probe is an *Eco*RI-*Hin*dIII fragment of the murine IFN-α4 and detects multiple IFN-α subtypes (16).

Results

Cytokine production of splenic DCs in response to CpG DNAs

We first evaluated CpG DNA-induced production of cytokines in splenic CD11c$^+$ DCs (Fig. 1*A*). D19 and ODN1668 were chosen as representatives of A/D-type and conventional CpG DNAs, respectively. Both increased the production of TNF-α and IL-12 p40 from splenic DCs in a dose-dependent manner, although D19 needed higher concentrations than ODN1668 to achieve comparable levels of cytokine production (Fig. 1).

We next measured IFN-α production of CpG DNA-stimulated splenic DCs. D19-induced IFN-α secretion was detected above a concentration of 0.1 μM and increased in a dose-dependent manner. ODN1668 could also induce IFN-α, but the production was only detected at concentrations of 0.01–0.3 μM and was abolished at higher concentrations. Furthermore, the maximal induction was much less than that of D19-stimulated DCs. Thus, ODN1668 and D19 have differential activities on mouse DCs in terms of IFN-α production.

In the presence of Flt3L, CD11c$^+$ cells, including IFN-α-producing cells, can be generated in vitro from BM cells (14). We next tested the responses of Flt3L-BMDCs to D19 and ODN1668.

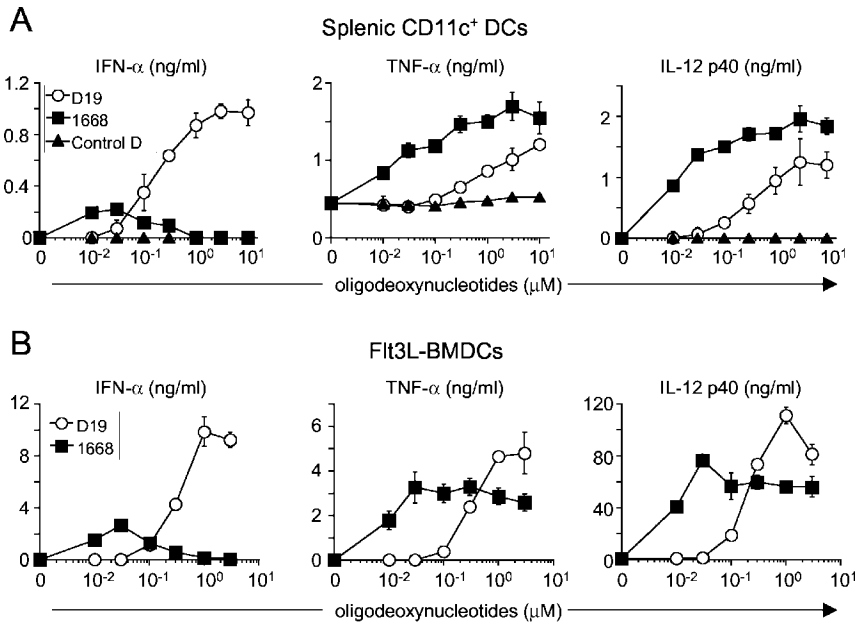

FIGURE 1. Cytokine production by splenic CD11c$^+$ cells (*A*) and Flt3L-BM DCs (*B*) in response to CpG DNAs. Splenic CD11c$^+$ cells were enriched by MACS and stimulated with the indicated concentrations of synthetic oligodeoxynucleotides for 24 h. Control D carries GpC instead of the CpG dinucleotide sequence of D19. Concentrations of IFN-α, TNF-α, and IL-12 p40 in the culture supernatants were measured by ELISA. Data are shown as the mean ± SD.

These DCs could also produce TNF-α and IL-12p40 in response to D19 or ODN1668. Similar to splenic DCs, Flt3L-BMDCs also responded better to ODN1668 than to D19. D19 could induce Flt3L-BMDCs to produce IFN-α in a dose-dependent manner, while ODN1668 induced small amounts of IFN-α only at 0.01–0.3 μM. Taken together, not only splenic CD11c$^+$, but also in vitro DCs, differentially responded to the two types of CpG DNAs in a similar manner.

B220$^+$ CD11c$^+$ cells are major IFN-α-producing cells in response to D19

DCs can be divided into subsets according to their surface molecule expression profiles (17). Among splenic DC subsets, B220$^+$CD11c^{dull} cells show a unique ability to produce IFN-α upon viral infection (18–21). To characterize the cell population involved in cytokine production from CpG DNA-stimulated splenic DCs, we purified B220$^+$CD11c^{dull} and B220$^-$CD11c^{high} cells by cell sorting and analyzed their responses to ODN1668 or D19 (Fig. 2*A*). In response to ODN1668, both B220$^+$CD11c^{dull} and B220$^-$CD11c^{high} cells produced IL-12 and enhanced their surface expression of CD40 and CD86. Neither population produced detectable levels of IFN-α in response to 3 μM ODN1668. In the case of D19 stimulation, both populations showed increased expression of costimulatory molecules. However, B220$^-$CD11c^{high} cells produced IL-12, but not IFN-α, whereas B220$^+$CD11c^{dull} cells produced IFN-α, but not IL-12.

Flt3L-BMDCs can also be divided into two subsets according to B220 expression (14). We next tested the responses of these subpopulations to CpG DNAs. The B220$^+$ and B220$^-$ cells in Flt3L-BMDCs responded to the two types of CpG DNAs in a manner similar to that of splenic B220$^+$CD11c^{dull} and B220$^-$CD11c^{high} cells, respectively (Fig. 2*B*). Thus, ODN1668 could activate both populations indistinguishably, whereas D19 showed differential cytokine-inducing ability in the two populations.

IFN-α production in response to D19 is dependent on TLR9 and MyD88

TLR9 or its cytoplasmic adapter, MyD88, is essential for conventional CpG DNA signaling (8, 22, 23). We next investigated whether the two types of CpG DNAs show any differences in their dependency on TLR9 or MyD88. Among splenocytes, CD11c$^+$ cells were mainly involved in IFN-α production in response to D19 (Fig. 3*A*). This IFN-α production was completely abolished in the absence of TLR9 or MyD88. Furthermore, whole and CD11c$^+$ splenocytes from TLR9$^{-/-}$ or MyD88$^{-/-}$ mice lacked both ODN1668- and D19-induced IL-12 production (Fig. 3*A*).

Next, we investigated the cytokine-producing ability of CpG DNA-stimulated Flt3L-BMDCs. IL-12 production in response to both types of CpG DNAs was abolished in Flt3L-BMDCs derived from both TLR9$^{-/-}$ and MyD88$^{-/-}$ mice (Fig. 3*B*). Furthermore, IFN-α production in response to D19 as well as that to 0.03 μM ODN1668 were completely abolished in the mutant Flt3L-BMDCs. Thus, the ability of both conventional and A/D-type CpG DNAs to induce cytokine production from in vivo and in vitro DCs was dependent on TLR9 and MyD88.

DNA-PKcs is not essential for responses to CpG DNA

It has been reported that DNA-PKcs is necessary for cytokine production in response to CpG DNAs, because DNA-PKcs$^{-/-}$ cells lacked the response (24). We next examined cytokine production of DNA-PKcs$^{-/-}$ cells in response to CpG DNAs. DNA-PKcs$^{-/-}$ mice lack mature B and T cell population due to defective DNA recombination (12, 25). Therefore, CD11c$^+$ cells were isolated by MACS from wild-type and mutant splenocytes. Splenic CD11c$^+$ cells were stimulated with D19 or ODN1668 for 24 h and analyzed for their cytokine production (Fig. 4*A*). DNA-PKcs$^{-/-}$ CD11c$^+$ cells retained the ability to produce IFN-α and IL-12 in response to CpG DNAs.

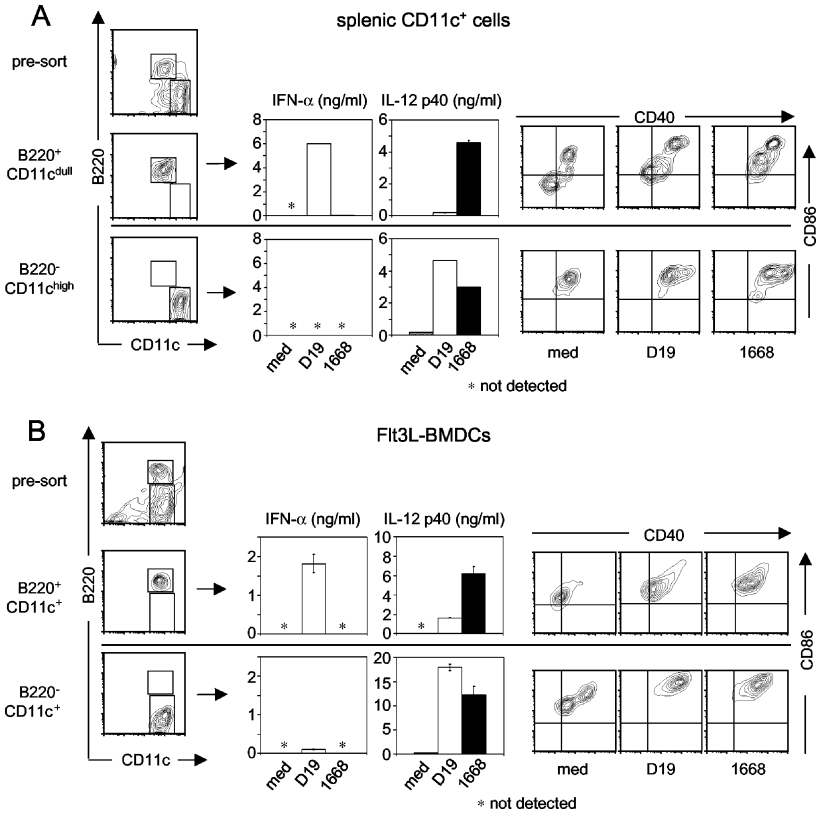

FIGURE 2. B220$^+$CD11c$^+$ and B220$^-$CD11c$^+$ cells differentially respond to two types of CpG DNAs. Splenic B220$^+$CD11c^{dull} and B220$^-$CD11c^{high} cells (*A*) were purified by FACS. Sorted cells were cultured at 4×10^4 cells/well with or without 3 μM CpG DNAs for 24 h. Concentrations of IFN-α or IL-12 p40 were measured by ELISA and are shown as the mean $\pm$ SD. Surface expression of costimulatory molecules was analyzed by flow cytometry. Flt3L-BMDCs (*B*) were also sorted by B220 expression and analyzed similarly to splenic DCs. Reanalysis of sorted cells verified that >95% cells exhibited the expected FACS profiles.

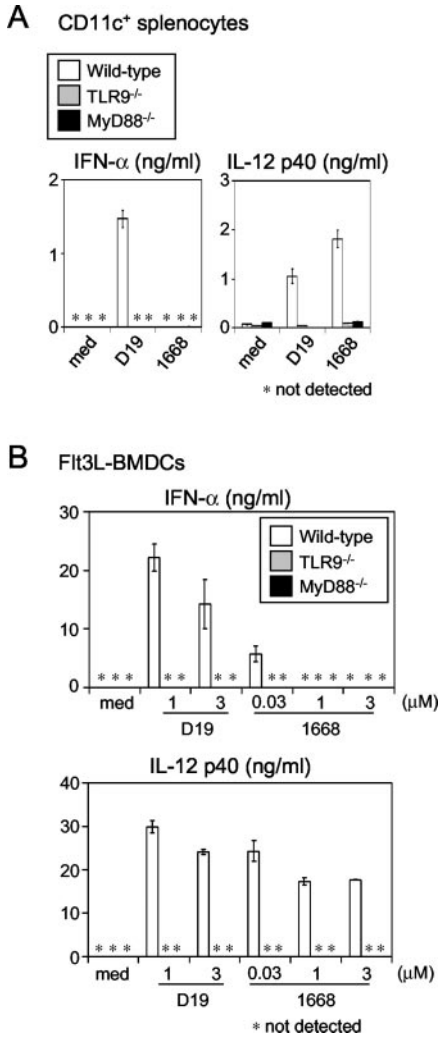

FIGURE 3. TLR9$^{-/-}$ or MyD88$^{-/-}$ cells lack any cytokine production in response to either type of CpG DNA. Splenic CD11c^{+} DCs (*A*) and Flt3L-BMDCs (*B*) were prepared from wild-type, TLR9$^{-/-}$, or MyD88$^{-/-}$ mice. Cells were stimulated with the indicated concentrations of D19 or ODN1668 for 24 h. Concentrations of IFN-α and IL-12 p40 were measured by ELISA. Data are shown as the mean ± SD of one representative experiment.

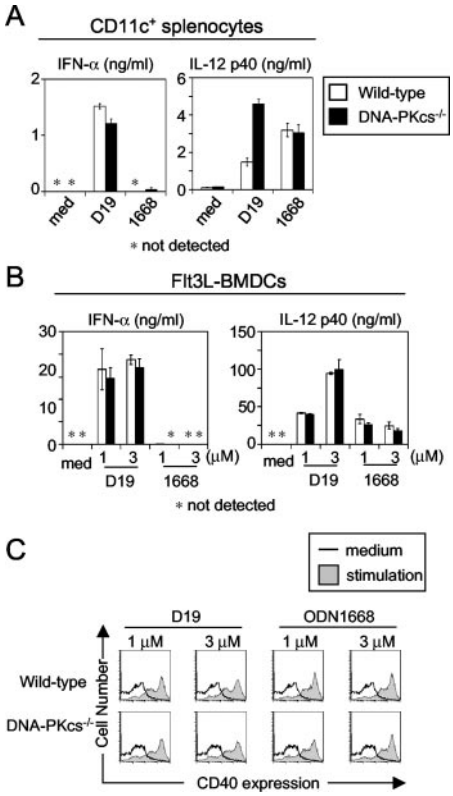

FIGURE 4. DNA-PKcs$^{-/-}$ cells normally respond to CpG DNAs. Splenic CD11c^{+} DCs (*A*) and Flt3L-BMDCs (*B*) from wild-type or DNA-PKcs$^{-/-}$ mice were stimulated with 3 μM D19 or ODN1668 (1668) for 24 h. Concentrations of IFN-α and IL-12 p40 in the culture supernatants were measured by ELISA. Data are shown as the mean ± SD. Surface expression of CD40 on Flt3L-BMDCs was analyzed by flow cytometry (*C*). Similar results were obtained from three independent experiments.

We also analyzed the effects of both CpG DNAs on wild-type and DNA-PKcs$^{-/-}$ Flt3L-BMDCs. FACS analysis revealed that the population ratio of B220^{+}CD11c^{+} and B220^{-}CD11c^{+} cells in Flt3L-BMDCs from DNA-PKcs$^{-/-}$ mice was comparable to that from wild-type mice (data not shown). DNA-PKcs$^{-/-}$ Flt3L-BMDCs augmented IL-12 production and surface expression of CD40 and CD86 in response to ODN1668 (Fig. 4, *B* and *C*, and data not shown). These responses were also observed in D19-stimulated DNA-PKcs$^{-/-}$ Flt3L-BMDCs. Furthermore, the mutant BMDCs produced IFN-α in response to D19 (Fig. 4*B*). Thus, these results clearly indicated that DNA-PKcs are dispensable for all immunostimulatory effects of both types of CpG DNAs.

Northern blot analysis of CpG DNA-stimulated DCs

IRF7 expression is induced by type I IFNs or viral infection and involved in type I IFN production (16, 26, 27). Therefore, we examined whether the IRF7 mRNA is differentially induced by the two types of CpG DNAs. As shown in Fig. 5*A*, IRF7 mRNA induction was observed in D19-stimulated Flt3L-DCs. ODN1668 also up-regulated IRF7 mRNA expression at both low and high concentrations with similar kinetics and levels as D19, although induction of IFN-α and IFN-β mRNA was abolished in DCs stimulated with high concentrations of ODN1668.

It has been reported that in viral infections, IRF7 mRNA induction is dependent on IFN-α/β (27). Therefore, we analyzed gene expression of CpG DNA-stimulated IFN-α/βR-deficient (IFN-α/βR$^{-/-}$) DCs. IL-12p40 mRNA induction by both CpG DNAs was retained in IFN-α/βR$^{-/-}$ DCs (Fig. 5*B*). However, IFN-α/β mRNA up-regulation by D19 was abolished in the absence of IFN-α/βR (Fig. 5*B*), indicating the critical involvement of type I IFN signaling in type I IFN production induced by D19. Furthermore, IRF7 mRNA induction by D19 as well as by ODN1668 was abolished in the absence of IFN-α/βR. This suggests that type I IFN signaling is essential for IRF7 mRNA induction even in ODN1668-stimulated DCs. These data, however, indicate that the loss of IFN-α mRNA induction at higher doses of ODN1668 may not be due to a decrease in IRF7 induction.

Discussion

In this study we analyzed cellular and molecular basis for the actions of two types of CpG DNAs. Conventional CpG DNAs can activate murine DCs to produce IL-12 and enhance the surface expression of costimulatory molecules. A/D-type CpG DNAs can also activate murine DCs in a similar manner as conventional CpG DNAs and, moreover, can induce IFN-α production from CD11c^{+} cells. Splenic CD11c^{+} cells can be divided into two populations, B220^{+}CD11c^{dull} and B220^{-}CD11c^{high} cells. The former cells were identified as IFN-α-producing cells during viral infection

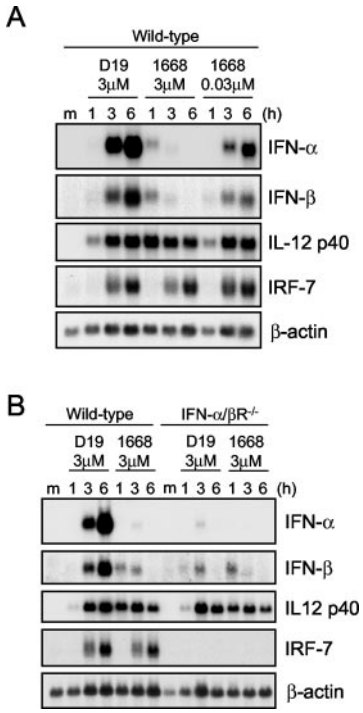

FIGURE 5. Northern blot analysis of Flt3L-BMDCs stimulated with D19 or ODN1668. Flt3L-BMDCs from wild-type or IFN-$\alpha/\beta R^{-/-}$ mice were stimulated with CpG DNAs for the indicated periods. Total RNAs were extracted and subjected to Northern blot analysis.

(18–20). We found that A/D-type CpG DNAs also stimulated $B220^{+}CD11c^{dull}$ cells to induce IFN-α production. $B220^{+}CD11c^{dull}$ cells could also produce significant, but smaller, amounts of IFN-α by conventional CpG DNAs only at low doses, while $B220^{-}CD11c^{high}$ cells did not (data not shown). Interestingly, although $B220^{-}CD11c^{high}$ cells secreted similar amounts of IL-12 in response to the two types of CpG DNAs, $B220^{+}CD11c^{dull}$ cells produced smaller amounts of IL-12 when stimulated with A/D-type CpG DNAs than with conventional CpG DNAs. Meanwhile, costimulatory molecule expression in $B220^{+}CD11c^{dull}$ and $B220^{-}CD11c^{high}$ cells was comparatively up-regulated by both types of CpG DNAs. These results suggest that differential responses to conventional or A/D-type CpG DNAs are observed in cytokine-producing ability, but not in the ability to enhance costimulatory molecule expression. Notably, $B220^{-}CD11c^{high}$ cells, which cannot produce IFN-α, responded to the two types of CpG DNAs in an indistinguishable manner. Thus, the differential ability of DCs to produce cytokines in response to conventional or A/D-type CpG DNAs is based on the characteristics of $B220^{+}CD11c^{dull}$ cells.

At present we do not know how TLR9 can transduce such differential activity depending on the ligand. Klinman et al. (7) have shown that TLR9 overexpression can render a human kidney cell line responsive to conventional CpG DNAs, but not to A/D-type CpG DNAs. This suggests that TLR9 is not sufficient for transducing the A/D-type CpG DNA signal, and that another molecule(s) cooperatively functions to induce IFN-α production with TLR9. It is assumed that oligodeoxynucleotides are incorporated into cells through a pathway not requiring any specific sequences and that only CpG DNAs can bind to TLR9 and trigger TLR9 signaling in the endosome (10, 28). However, confocal microscopic analysis revealed that conventional and A/D-CpG DNAs are destined for different cell compartments (29). Such differential behavior of CpG DNAs might lead to the induction of distinct cytokines. It is also possible that differential TLR9 responses are caused by coligation of other transmembrane proteins. In this context, it is noteworthy that scavenger receptor A (SR-A) can bind to the polyG stretch and that SR-A ligands can inhibit the binding of CpG DNAs containing the polyG stretch to splenic $CD11c^{+}$ DCs (30). However, it remains unknown whether SR-A is involved in DC responses caused by A/D-type CpG DNAs.

DNA-PKcs was suggested as a signaling molecule for CpG DNA-induced immune responses (24). However, a recent report has shown that DNA-PKcs is not essential for CpG DNA responses (31). Our present results are consistent with the latter report and further show that the enzyme is dispensable not only for conventional, but also for A/D-type, CpG DNAs-induced signaling. In addition, $MyD88^{-/-}$ and $TLR9^{-/-}$ cells lacked any response to either type of CpG DNAs (Fig. 3). Thus, CpG DNAs can manifest their multiple immunostimulatory functions through the TLR9-MyD88-dependent pathway.

Viral infection can induce type I IFN production that is vigorously amplified by type I IFN itself (32, 33). IFN-$\alpha/\beta R^{-/-}$ cells decreased their ability to produce IFN-α in response to A/D-type CpG DNAs, indicating that type I IFN signaling is also involved in TLR9-induced IFN-α production. IRF7 mRNA can be induced by type I IFN. The induction was suggested to be critical for IFN-α production through the analysis of IRF-9-deficient mice that lack IRF7 mRNA induction (27). However, wild-type DCs could produce similarly increased levels of IRF7 mRNA expression in response to 3 μM ODN1668, although IFN-α production was profoundly decreased (Fig. 5). Thus, IRF7 mRNA induction is not sufficient for TLR9-induced IFN-α production.

In response to 3 μM ODN1668, low levels of type I IFN mRNA expression can be induced at 1 h, but the induction rapidly declines at later time points. Meanwhile, at 0.03 μM ODN1668 the induction comes later and reaches comparable levels as with D19. These results suggest the possibility that a high concentration of ODN1668 induces unknown negative feedback mechanism resulting in the abolishment of type I IFN gene expression. Further studies are necessary to clarify how TLR9 induces differential responses depending on its ligands.

Among TLR family members, TLR7 is closely related to TLR9 based on their molecular structures. In humans, TLR7 and TLR9 are expressed on a subset of DCs, PDC. Activation of TLR7 or TLR9 enhances the survival of DCs and the expression of surface molecules, such as costimulatory molecules and MHC class II. Thus, TLR7 and TLR9 have common features in their molecular structures and functions. While TLR9 is expressed exclusively on PDC, TLR7 is also expressed on another subset, myeloid DC. Experiments with TLR7 ligands clarified that TLR7 signaling can differentially induce IFN-α and IL-12 from PDC and myeloid DC, respectively (34). The mechanism is unknown at present, but it is intriguing that murine TLR9 and the human TLR7 systems are well conserved in differential cytokine inducibility depending on DC subsets. Another intriguing point is that murine $B220^{+}CD11c^{dull}$ cells do not produce high levels of IL-12 in response to D19, although they can in response to ODN1668. This is in contrast to the human TLR7 system, because human PDC cannot produce IL-12 in response to any stimuli. IFN-α can inhibit IL-12 production (35), but that cannot account for the inability of A/D-type CpG DNA-stimulated $B220^{+}CD11c^{dull}$ cells to produce IL-12, because costimulation with IFN-α and ODN1668 did not decrease IL-12 production from $B220^{-}CD11c^{high}$ cells (data not shown). Furthermore, type I IFN-neutralizing Abs could not increase IL-12 production from D19-treated $B220^{+}CD11c^{dull}$ cells (data not shown). Thus, it is unlikely that IFN-α production induced by D19 prevents $B220^{+}CD11c^{dull}$ cells from producing IL-12.

The present study has revealed subset-dependent responses of murine DCs to distinct types of CpG DNA. Further clarification of the underlying molecular mechanisms should contribute to elucidating how DCs are activated by CpG DNAs and to developing efficient vaccination strategies with CpG DNAs.

Acknowledgments

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