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Vaccination with Plasmid DNA Activates Dendritic Cells via Toll-Like Receptor 9 (TLR9) but Functions in TLR9-Deficient Mice

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We analyzed whether the immunobiology of vaccinating plasmid DNA containing a transcription unit for OVA is influenced by immunostimulatory CpG motifs in the plasmid backbone. Indeed, plasmid DNA differentially activated in vitro myeloid and plasmacytoid dendritic cells (DCs) provided they expressed the CpG-DNA receptor, Toll-like receptor 9 (TLR9). Dependent on the DC subset, activation resulted in type 1 IFN production, while both DC subsets produced IL-6 and up-regulated expression of costimulatory molecules CD40 and CD86. In vivo, however, even upon repeated vaccination with plasmid DNA, priming of OVA-specific CTL and clonal expansion of SIINFEKL-specific CD8 T cells were equal in TLR9-positive and TLR9- or MyD88-negative mice. Overall, these results negate a dominant role of CpG-DNA/TLR9 interactions in long-term vaccination protocols. The Journal of Immunology, 2003, 171: 5908–5912.

Vaccination with naked DNA containing the gene for the Ag of interest is under intensive investigation, because both cellular and humoral immune responses become induced (1, 2). Several factors are likely to influence the magnitude of immune response generated, including optimal gene expression and the immunogenicity of the expressed gene (1, 2). The discovery of immunostimulatory effects of genomic bacterial DNA (3) mimicked by unmethylated CpG motifs within synthetic oligodeoxynucleotides (CpG-ODN) (4) (4) led to the conclusion that the adjuvant properties of bacterial plasmid DNA (pDNA) are dependent on their content of CpG motifs (5, 6). Indeed, there is compelling evidence that the immunogenicity of pDNA is influenced by CpG motifs contained within the plasmid backbone, e.g., within the antibiotic resistance gene and the noncoding region of the plasmid (7, 8).

Recently, it has been demonstrated that Toll-like receptor 9 (TLR9) is essential for CpG-ODN-induced innate immune cell activation, because TLR9-deficient mice are refractory (9). Furthermore, TLR9 expression is sufficient to confer responsiveness to CpG-ODN (10). However, because there are structural differences between dsDNA and immunostimulatory ssCpG-ODN, the cellular and molecular mechanisms of how pDNA activates innate immune cells such as macrophages and dendritic cells (DCs) remain to be defined.

In this study, we investigated whether naked pDNA containing a transcription unit for OVA activates murine B cells and DCs of the plasmacytoid and myeloid lineage via TLR9. We show that pDNA lacks mitogenicity for B cells, yet requires TLR9 to activate plasmacytoid and myeloid DC subsets. Unexpectedly, however, pDNA vaccination of TLR9-deficient mice as well as of MyD88-deficient mice resulted in robust priming and clonal expansion of OVA-specific CTLs.

Materials and Methods

Mice
C57BL/6 mice were purchased from Harlan Winkelmann (Borchen, Germany). TLR9- and MyD88-deficient mice were kindly provided by S. Akira (Osaka, Japan) and bred and backcrossed to C57BL/6 (sixth backcross) in house. All animals were housed under specific pathogen-free conditions and used at 8–12 wk of age.

Cell lines and in vitro culture medium
The EL-4 (H-2b) thymoma cell line was purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 IU/ml penicillin G, 100 IU/ml streptomycin sulfate (Biochrom, Berlin, Germany), and 50 μmol/L 2-ME (Life Technologies, Karlsruhe, Germany) at 37°C and 5% CO2.

Reagents
The imidazoquinoline R848 was a gift of Coley Pharmaceuticals (Hilden, Germany). Chicken egg albumin (OVA), Escherichia coli-derived LPS, poly(I:C), and tripalmitoyl-cystein-seryl-(lysyl)3-lysine (Pam3Cys) were purchased from Sigma-Aldrich (Taufkirchen, Germany). The peptide SIINFEKL (OVA peptide 257–264) was custom synthesized by Research Genetics (Huntsville, AL). Phosphorothioate-stabilized ODN were synthesized by MWG (Ebersberg, Germany). CpG-ODN 1668 entails the sequence: 5′–TCCATGACGTTCCTGATGCT–3′. CpG-ODN 2216 contains the sequence GCCGGACGATCGCTGGGGG. Only the underlined poly(G) runs at 5′ and 3′ were thioate stabilized.

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5 Abbreviations used in this paper: ODN, oligodeoxynucleotide; BMDC, bone marrow-derived DC; DC, dendritic cell; Flt3L, Flt3 ligand; Pam3Cys, tripalmitoyl-cystein-seryl-(lysyl)3-lysine; pDC, plasmacytoid-like DC; pDNA, plasmid DNA; TLR, Toll-like receptor; wt, wild type.
Plasmid

cpOVA plasmid vector consists of a pcDNA3 backbone and an OVA-encoding insert. The plasmid has a CMV promoter and an ampicillin resistance gene. The plasmid was amplified using Qiagen (Hilden, Germany) EndoFree Plasmid Mega kit, according to the manufacturer’s protocol.

Immunization and chromium release assay

Priming for OVA-specific CTLs was induced by injection of 0.2 mg of pcOVA in 100 μl of PBS s.c. at the tailbase of mice. On days 10 and 20, boost injection of the same amount was performed. Seven days after last injection, spleens were removed, and single-cell suspensions were prepared (5 × 10^6/ml) and cultured with SIINFEKL-labeled (0.5 μM) irradiated (15 Gy) spleen cells (2 × 10^6/ml, derived from C57BL/6 mice) plus 10 U/ml rIL-2. At day 7, the ^51Cr release assay was performed, as described (11).

Specific lysis was calculated according to the formula: percent specific lysis = (cpm target − cpm spontaneous release) / cpm maximum release − cpm spontaneous release) × 100. Spontaneous release ranged between 5 and 15%.

Ex vivo tetramer staining of primed SIINFEKL-specific CD8 T cells

As described (12), splenic cell suspensions were depleted for RBC, followed by double staining with anti-CD8 APC (clone CD8a; Caltag Laboratories, Burlingame, CA) and MHC SIINFEKL tetramer PE (H-2Kb/murine β2-microglobulin streptavidin-PE) for 1 h at 4°C. Additionally, an Fe block (CD16/CD32, 2.4G2; BD Pharmingen, Heidelberg, Germany) was used to avoid unspecific Ab staining. For life/death cell discrimination, cells were subsequently incubated with ethidium monoazide bromide (Molecular Probes, Leiden, The Netherlands). At least 1 × 10^6 CD8-positive events were acquired on a FACScan flow cytometer (BD Biosciences, Heidelberg, Germany) and analyzed with FlowJo software (Tree Star, Ashland, OR). mAbs were from BD Pharmingen.

Preparation of B cells

Erythrocytes within single-cell suspensions of C57BL/6 spleens were ammonium-chloride lysed for 2 min on ice. Subsequently, cells were incubated with magnetic beads coated with the CD43 Ab (Miltenyi Biotec, Bergisch Gladbach, Germany). Untouched resting B cells (CD43low) were obtained by depletion of CD43-expressing cells using MS^+ separation columns (MiniMacs; Miltenyi Biotec).

Proliferation assay

B cell mitogenicity was determined after stimulation of 2 × 10^5/culture of purified B cells (derived from TLR9+/+ mice or wild-type (wt) C57BL/6 mice) with graded doses of pCpOVA plasmid or LPS (1 μg/ml) at 37°C in 5% CO2. After 72 h, [H]thymidine incorporation was determined 16 h later.

Flt3 ligand culture bone marrow-derived DCs (BMDC)

As described (13), bone marrow cells were cultured for 8–9 days with human Flt3 ligand (Flt3L; kindly donated by K. Shortman, Melbourne, Australia) plasmacytid-like DCs (pDCs), and conventional DCs were sorted using a MoFlo cell sorter using CD11c^{bsh} CD45RA^{high} and CD11b^{low} as selection criteria for pDCs. As selection criteria for conventional DCs, we used CD11c^{bsh}, CD45RA^{low}, and CD11b^{bsh} expression.

Detection of cytokines

Cytokines were detected by specific ELISAs for IFN-α from PBL Biochemical Laboratories, (Picataway, NJ) and donkey anti-rabbit HRP conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA), as described (14). IL-6 ELISA kits were from BD Biosciences.

Results

pDNA lacks mitogenicity toward purified B cells

Immunostimulatory ssCpG-ODN are mitogenic for murine B cells known to express TLR9 (4). Therefore, we first evaluated whether dsDNA exhibits mitogenic activity toward purified murine B cells, as does ssCpG-ODN 1668, or LPS. As shown in Fig. 1, at all concentrations tested, dsDNA failed to drive mitogenic responses both in wt and TLR9-deficient B cells. In contrast, low, but significant concentrations of IL-6, IL-12 p40, and IL-10 were detectable in the culture supernatants, indicating inefficient activation of B cells by dsDNA (data not shown). As expected, the mitogenicity of CpG-ODN 1668 was dependent on TLR9 expression, while that of the TLR4 ligand LPS (15) was not.

pDNA activates DCs via TLR9

In the presence of Flt3L, CD11c-positive DCs can be generated in vitro from bone marrow cells, including a subset of IFN-α-producing pDC (16). Unlike human pDCs, which may be better activated by conventional B type CpG-ODNs compared to A type CpG-ODNs (17, 18), mouse pDC become activated by both CpG-ODN types in a TLR9-dependent fashion (19, 20). It is also established that the antiviral molecule R848 (Imiquimod) activates DCs via TLR7 (21), Pam3Cys via TLR2, and poly(I:C) via TLR3 (22). Therefore, we compared the responsiveness of Flt3L-BMDCs from wt mice and TLR9-deficient mice to dsDNA, the conventional ssCpG-ODN 1668, the A type ssCpG-ODN 2216, R848, LPS, Pam3Cys, and poly(I:C) via TLR3 (22). Therefore, we compared the responsiveness of Flt3L-BMDCs from wt mice and TLR9-deficient mice to dsDNA, the conventional ssCpG-ODN 1668, the A type ssCpG-ODN 2216, R848, LPS, Pam3Cys, and poly(I:C), respectively. Flt3-BMDCs from wt mice, but not from TLR9−/− produced IFN-α only in response to A type CpG-ODN 2216 and to pDNA, albeit pDNA was less effective (Fig. 2A). In contrast, all TLR ligands as well as pDNA stimulated IL-6 production in wt mice-derived Flt3-BMDCs, yet TLR9−/− mice-derived Flt3-BMDCs were unresponsive to pDNA and CpG-ODNs (Fig. 2B). Furthermore, pDNA caused up-regulation of CD40, CD86, and CD69 as did CpG-ODN 1668 (data not shown, and see below).

pDNA activates pDCs and myeloid DCs via TLR9

Next, we generated Flt3L-BMDCs from wt mice and TLR9-deficient mice and purified CD45RA^{high}CD11b^{low}CD11c^{high} pDCs and CD45RA^{low}CD11b^{high}CD11c^{high} conventional DCs by cell sorting, respectively. In response to pDNA, wt mouse-derived and thus TLR9-positive pDCs produced IFN-α and IL-6, although A type CpG-ODN 2216 was more effective. In contrast, mouse-derived conventional DCs responded to pDNA with IL-6 production, but failed to produce α-IFNs. However, TLR9-deficient pDCs and conventional DCs were unresponsive (data not shown).

In parallel, we analyzed whether TLR9 expression by the two DC subsets was required for pDNA-driven up-regulation of CD40, CD86, and CD25. Cell surface up-regulation of these activation/costimulatory markers could clearly be observed in both wt mouse-derived pDC and conventional DC subsets (Fig. 3). However, TLR9-deficient DC subsets were refractory (Fig. 3). Of note,
The DC subsets were incubated with plasmid (200 U/ml, and for IL-6 was 16 pg/ml. Culture supernatants were analyzed for IFN-α (A) and IL-6 (B) by ELISA. The error bars indicate the variation of duplicate wells. The data are representative for >3 experiments. The sensitivity (ELISA) for IFN-α was 32 U/ml, and for IL-6 was 16 pg/ml.

FIGURE 2. Cytokine production of Flt3L-cultured BMDC (FL-BM) induced by plasmid is dependent on TLR9. A total of 1 × 10^5 FL-BM of wt (■) or TLR9 knockout (□) mice was incubated in 200 μl for 26 h with the indicated stimuli: Pam3Cys (1 μg/ml), LPS (1 μg/ml), R-848 (1 μg/ml), poly(I:C) (100 μg/ml), CpG-1668 (0.5 μM), CpG-2216 (0.5 μM), and pDNA (200 μg/ml). Culture supernatants were analyzed for IFN-α (A) and IL-6 (B) by ELISA. The error bars indicate the variation of duplicate wells. The data are representative for >3 experiments. The sensitivity (ELISA) for IFN-α was 32 U/ml, and for IL-6 was 16 pg/ml.

TLR9-deficient pDCs and conventional DCs up-regulated CD40, CD86, and CD25 in response to the TLR7 ligand R-848 (Fig. 3), implying that unresponsiveness toward pDNA is due to TLR9 deficiency.

pDNA vaccination functions in TLR9-deficient mice

It has been reported that CpG motifs within DNA vaccines augment the immunogenicity of the Ag transcribed (21, 23, 24). Perhaps the strongest evidence that CpG motifs contribute to the immunogenicity of DNA vaccines was provided by Sato et al. (7), who transfected human monocytes with immunostimulatory CpG-DNA. Interestingly, in the absence of pDCs, human monocytes are believed to be unresponsive to CpG motifs because they do not express TLR9 (25). In this study, we describe that pDNA activates via TLR9 both murine conventional DCs as well as pDCs (Fig. 3). Therefore, we expected that vaccination of TLR9-deficient mice or MyD88-deficient mice with pDNA containing a transcription unit for OVA is poor. However, priming of OVA-specific CTL turned out to be robust both in TLR9-positive and TLR9- or MyD88-negative mice (Fig. 4). Primed T cells were assayed in this study by using an in vitro restimulating step that may cloud quantitative differences in pDNA-vaccinated wt vs TLR9−/− or MyD88−/− mice. We therefore resorted to the tetramer technology to quantitate in parallel the clonal size of SIINFEKL-specific T cells in the spleen of mice tested for OVA-primed T cells (Fig. 5). Spleen cells of nonvaccinated wt mice (used as negative control) expressed ~0.03–0.2% of SIINFEKL tetramer-binding CD8 T cells. However, in plasmid vaccinated wt as well as TLR9−/− and/or MyD88−/− mice, the SIINFEKL tetramer-binding CD8 T cells had expanded up to 3–4%. We thus concluded that pDNA-mediated Ag-specific T cell priming as well as clonal size of expanded SIINFEKL-specific CD8 T cells were similar in wt as well as TLR9−/− or MyD88−/− mice.

Discussion

In this study, we analyzed whether pDNA containing a transcription unit for OVA activates DCs via TLR9. In addition, we asked whether TLR9 expression affects priming and clonal expansion of OVA-specific CTL. Although dsDNA failed to drive proliferation of murine B cells known to express TLR9, pDNA activated TLR9-positive conventional DCs or pDCs both in terms of cytokine production and enhanced expression of costimulatory molecules. Notably, A type ssCpG-DNA as well as dsDNA induced IFN-α by in vitro generated pDCs, albeit the latter was less efficient. Because TLR9-deficient DC subsets failed to respond to pDNA, we conclude that CpG motifs contained in bacterial dsDNA activate murine DCs via TLR9. These results given, we were surprised to
observe that pDNA vaccination of TLR9- or MyD88-deficient mice resulted in robust priming and clonal expansion of OVA-specific CTL, the magnitude of which appeared similar to that in wt mice. Because we restricted our analysis to priming and clonal expansion of CTL, there is now a need to analyze the breadth of humoral responses triggered in TLR9-deficient mice.

pDNA and immunostimulatory CpG-ODN differ structurally. For example, in most studies, ssCpG-ODN containing ~20 phosphorothiate-stabilized nucleotides have been used (reviewed in Refs. 5 and 6). Plasmids, however, have phosphodiester bonds, are double stranded, and entail an abundance of nucleotides likely to generate multiple CpG motifs. Similar to ssCpG-ODN, dspDNA also is taken up by macrophages and DCs, presumably via receptor-mediated endocytosis (26, 27), which appears not to involve class A or B scavenger receptors (28). In this study, we show that murine conventional and pDC subsets deficient in TLR9 are unresponsive to pDNA, both in terms of cytokine production and up-regulation of costimulatory molecules. In contrast, wt mouse-derived DC subsets are responsive, and pDCs even produce type 1 IFNs. We therefore conclude that pDNA uses TLR9 to initiate the Toll/IL-1 signaling pathway for activation of NF-κB and mitogen-activated protein kinases as does CpG-DNA (20). It is likely that upon endocytosis, pDNA becomes at least in part degraded by DNase II in the endosomal-lysosomal compartment (29) to liberate CpG motifs. Independently, immunostimulatory CpG-DNA has previously been shown to recruit, via TLR9, the MyD88 adaptor molecule to late endosomal organelles (30).

Given that immunostimulatory ssCpG-ODNs are mitogenic for B cells (4), the ability of dspDNA to stimulate DCs (Figs. 2 and 3), but not B cell proliferation (Fig. 1) may surprise. However, it has been described that genomic dsDNA from bacteria needs to be DNase II in the endosomal-lysosomal compartment (29) to liberate CpG motifs. Upon endocytosis, pDNA becomes at least in part degraded by DNase II in the endosomal-lysosomal compartment (29) to liberate CpG motifs. Independently, immunostimulatory CpG-DNA has previously been shown to recruit, via TLR9, the MyD88 adaptor molecule to late endosomal organelles (30).

Given that immunostimulatory ssCpG-ODNs are mitogenic for B cells (4), the ability of dspDNA to stimulate DCs (Figs. 2 and 3), but not B cell proliferation (Fig. 1) may surprise. However, it has been described that genomic dsDNA from bacteria needs to be denatured to reveal B cell mitogenicity (31). In accordance, dspDNA failed to drive B cell proliferation (Fig. 1), but pDNA triggered in wt B cells low cytokine production (IL-6, IL-12 p40, IL-10). Interestingly, B cells endocytose ~10-fold less efficient DNA compared with DCs. Furthermore, when thioate-stabilized ssCpG-DNA such as ODN1668 are converted into dsCpG-ODN, their B cell mitogenicity fairs off. In contrast, the immunostimulatory activity of ssCpG-ODN displaying phosphodiester bonds is TLR9 dependent, even when converted into dsCpG-ODN, yet inferior to that of pDNA (data not shown). We thus conclude that structural differences between dsDNA and thioate-stabilized ssCpG-DNA as well as insufficient cellular uptake explain, at least in part, the poor B cell stimulatory activity of dspDNA.

Given the observation that in vitro pDNA encoding for OVA activated DCs in a TLR9-dependent fashion, yet primed and clonally expanded OVA-specific CTL in TLR9-deficient mice, a role of other TLRs could be involved. To test this possibility, we also vaccinated MyD88-deficient mice. Again, no differences were noted in priming/clonal expansion of SIINFEKL-specific CTL (Figs. 4 and 5). These results excluded a role of other, MyD88-dependent TLRs.

In terms of DNA vaccination, one fascinating aspect entails the question as to how the transcribed Ag is processed and presented to T cells: direct presentation by transfected somatic cells or alternatively by transfected DCs (32, 33), the former requiring cross-presentation and cross-priming (34). Even though the immunogenicity of pDNA has been linked with the adjuvant properties of CpG motifs within plasmid backbone (21, 23, 24), MHC class II-deficient mice lacking CD4 Th cells are unable to generate a CTL response following DNA immunization (35, 36). Accordingly, MHC class II-restricted T cell help can be considered as a rate-limiting step for CTL priming by pDNA, the adjuvant effect of CpG motifs (37) within the plasmid backbone per se being not sufficient (36). Perhaps complementary to this view, we observed that pDNA encoding for OVA activated DCs via TLR9, but use of this plasmid for DNA vaccination of TLR9-deficient mice nevertheless resulted in robust T cell priming and clonal expansion of SIINFEKL tetramer-binding CD8 T cells. Most likely, the transient nature of CpG-DNA-driven DC activation (37), as opposed to the more stable in vivo function of DNA plasmids (1), may allow long-term Ag production, and thus TLR9-independent CD4 Th cell activation, which could explain why TLR9 deficiency is not rate limiting in the vaccination system used. It follows that pDNA-driven, TLR9-dependent activation of DCs as assayed in a short-term assay (10–16 h) ought not directly be compared with yet poorly understood CD4 T cell activation events occurring during the long-term protocol used for DNA vaccination (3 × injections over 4 wk). We propose that within the time frame needed for repeated DNA vaccination (3–4 wk), MHC class II-restricted Th cells become activated by pathways not involving CpG motifs-TLR9 interactions. Once activated, Th cells may trigger OVA cross-presenting DCs via CD40-CD40 ligand interactions to become professional APCs able to cross-prime naive CD8 T cells.

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References
VACCINATION WITH pDNA ACTIVATES DCs


