Cutting Edge: Toll-Like Receptor 9 Expression Is Not Required for CpG DNA-Aided Cross-Presentation of DNA-Conjugated Antigens but Essential for Cross-Primming of CD8 T Cells

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References

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**CUTTING EDGE**

**Cutting Edge: Toll-Like Receptor 9 Expression Is Not Required for CpG DNA-Aided Cross-Presentation of DNA-Conjugated Antigens but Essential for Cross-Priming of CD8 T Cells**

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Covalent linkage of immunostimulatory CpG DNA to OVA results in CpG DNA-aided cross-presentation of OVA by dendritic cells (DCs). In vivo, cross-presentation is conditional for cross-priming of OVA-specific CD8 T cells. In this study, we investigated the involvement of the CpG DNA receptor Toll-like receptor (TLR9) in CpG DNA-aided cross-presentation and cross-priming. Although CpG DNA-aided cross-presentation is not altered in TLR9-deficient cells, TLR9 is required for maturation of APC allowing cross-priming, as resulting in CTL function. These findings imply that TLR9 does not trigger endocytosis of CpG-OVA conjugates, but activates DCs downstream of endocytosis. The Journal of Immunology, 2003, 170: 2802–2805.

**Materials and Methods**

C57BL/6 mice were purchased from Harlan Winkelmann (Borchsen, Germany). TLR9−/− mice were a gift from Dr. S. Akira (Osaka, Japan). All animals were kept under specific pathogen-free conditions and were used at 8–12 wk of age.

**Cell lines and in vitro culture medium**

EL-4 (H-2b) thymoma cells were purchased from the American Type Culture Collection (Manassas, VA). B3Z, a somatic T cell hybrid generated by fusing the OVA/Kb-specific cytotoxic clone B3 with a lacZ-inducible derivative of BWS147 fusion partner (14), was kindly provided by Dr. B. Kelsall (National institutes of Health, Bethesda, MD). Cells were cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin G, 100 μg/ml streptomycin sulfate (all from Biochrom, Berlin, Germany) and 50 μM l-2 ME (Life Technologies, Karlsruhe, Germany) at 37°C and 5% CO2.

**Generation of GM-CSF- and Flt3 ligand-cultured DC from bone marrow**

Flt3 ligand-supplemented bone marrow cell cultures were generated as described (9), and cells were used at 10 days of culture. Generation of GM-CSF-induced, bone marrow-derived DC cultures was performed as previously described (15). Cells were used at days 5–7.

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3 Abbreviations used in this paper: DC, dendritic cell; TLR, Toll-like receptor; ODN, oligodeoxynucleotide; S-MBS, sulfo maleimidobenzyloxymethyl hydroxyuccinimide ester; LN, lymph node; wt, wild type.
Reagents

Chicken egg albumin (OVA) was from Sigma-Aldrich (Taufkirchen, Germany). The peptide SIINFEKL (OVA peptide 257–264) was custom synthesized by Research Genetics (Huntsville, AL). FITC-labeled OVA was purchased from Molecular Probes (Leiden, The Netherlands).

Phosphothioate-modified immunostimulatory CpG DNA was custom synthesized by MWG (Ebersberg, Germany). The phosphothioated sulfhydryl-modified oligodeoxynucleotides (ODN; TriLink Biotechnologies, La Jolla, CA) used throughout this study consisted of 20 bases and contained a CpG motif (1668: 5′-S-TCCAGAACGTTGCCATGCT-3′). OVA was incubated with the cross-linker sulfo-maleimidobenzoyl-N-hydroxysuccinimide ester (S-MBS; Pierce, Bonn, Germany) in a 50 mM EDTA-PBS buffer (pH 7.0) at a molar ratio of 1:10 for 1 h at room temperature. The sulfhydryl-modified ODN were reduced in a 50 mM 1,4-DTT-PBS solution. Subsequently unbound S-MBS and 1,4-DTT were removed by chromatography on a Bio-Rad P-6 gel column (Bio-Rad, Munich, Germany). The activated ODN were incubated with the linker-modified OVA at a molar ratio of 5:1 for 2.5 h at room temperature and thereafter 1-cysteine was added to quench reactive S-MBS. Free ODN were removed by chromatography on a Superdex 75HR column (Amersham Biosciences, Freiburg, Germany). Purified conjugates were analyzed on a 6–20% gradient SDS-PAGE and consecutively silver-stained. To determine the ratio of bound ODN on OVA, a 4–15% gradient nondenaturing, nonreducing PAGE was run and silver-stained or visualized using ethidium bromide staining. Protein concentration was determined by the Lowry method (Pierce).

Purified conjugates were analyzed on a 15% gradient nondenaturing, nonreducing PAGE was run and silver-stained or visualized using ethidium bromide staining. Protein concentration was determined by the Lowry method (Pierce). The batches of CpG-OVA conjugates used in this study had a ratio of 2.5 CpG molecules linked to one OVA molecule.

Escherichia coli-derived LPS was purchased from Sigma-Aldrich.

**Immunization of chromatin release assay**

For induction of CTL, OVA and adjuvant were injected into both hind footpads of mice. Four days later, draining lymph nodes (LN) were removed and a single-cell suspension was prepared. LN cells (3 × 10⁶ cells/ml) were cultured for an additional 4 days in medium conditioned with 10 IU/ml rIL-2. The chromium release assay was performed as described (13). SIINFEKL peptide-untreated cells served for specificity control. Specific lysis was calculated according to the formula: percent specific lysis = (cpm_sample – cpm_spontaneous release) / maximum release – cpm_spontaneous release × 100.

**Uptake and activation analysis, mAbs**

To examine uptake of FITC-labeled CpG-OVA in vitro by bone marrow-derived Flt3 DC, cells were exposed to FITC-labeled OVA, mixed with CpG DNA 1668 or 1668-OVA-FITC conjugates (0.5 h at 37°C), washed with ice-cold 2% FCS-PBS containing 2 mM EDTA, and stained with the allophycocyanin-labeled anti-CD11c (clone HL3).

To analyze activation of DC, GM-CSF-cultured bone marrow-derived DC (15) were incubated with 10 μg/ml OVA conjugated with 1.14 μM CpG

![FIGURE 1. TLR9 is not required for uptake of OVA-CpG DNA conjugates in primary DC. Flt3 ligand-cultured bone marrow-derived DC from wt C57/B6 or TLR9−/− C57/B6 mice were incubated with 0.5 μg/ml OVA-FITC alone, mixed or conjugated with the CpG DNA 1668 (22.5 nM) for 30 min at 37°C, stained with allophycocyanin-labeled anti-CD11c, and analyzed by FACS. Percent FITC, and allophycocyanin double-positive, Ag-bearing CD11c+ DC are shown. The data are representative of three independent experiments.](image-url)

![FIGURE 2. Ag presentation by DC’s is not altered by the absence of TLR9. GM-CSF-derived bone marrow-derived DCs were incubated with 5 μg of OVA alone (Ova), mixed with CpG DNA 1668 (1668+Ova), or conjugated with 0.28 μM CpG DNA 1668 (1668-Ova) for 18 h. SIINFEKL presentation was assayed via B3Z hybridoma cells expressing an OVA-specific TCR via induction of β-galactosidase upon TCR engagement. Mean and SD of three wells is shown.](image-url)

![FIGURE 3. a. Up-regulation of costimulatory molecules is dependent on TLR9. Bone marrow-derived DCs from either wt or TLR9−/− mice were stimulated with CpG DNA 1668 conjugated to OVA (1668-Ova) (black line), LPS (dotted line), or medium only (filled) for 24 h at 37°C. Cells were washed two times, stained with anti-CD40 or anti-CD86 mAbs, and subjected to FACS analysis. b, TLR9 presence is essential for IL12 p40 and TNF-α secretion. Supernatants of stimulated cells (a) with CpG DNA 1668 conjugated to OVA (1668-Ova), LPS, or medium only, were collected and determined in triplicate by commercially available (IL12 p40; TNF-α) ELISA kits. Mean and SD are shown. Similar results were obtained in two additional experiments.](image-url)
CD8 T cell peptide SIINFEKL, functioned equally well in TLR9<sup>−/−</sup> or wt DCs (Fig. 2). We conclude that CpG DNA aided enhanced OVA uptake and processing, and SIINFEKL loading to MHC class I molecules functioned in the absence of TLR9.

Next, we addressed the issue of whether immunostimulatory CpG DNA covalently linked to OVA is effective in activating cross-presenting TLR9-deficient DCs into professional APCs. As shown in Fig. 3a, wt but not TLR9-deficient DCs up-regulated CD40 and CD80 and secreted IL-12 p40 and TNF-α (Fig. 3b) upon in vitro uptake of immunostimulatory CpG-OVA conjugates. Of note, the DC activation power of CpG DNA linked to protein (Fig. 3) was somehow reduced compared with free CpG DNA (9). Whether processing of OVA when linked to CpG DNA is still TAP-dependent has not yet been analyzed. However, in general, DCs of TLR9<sup>−/−</sup> were fully able to up-regulate CD40 and CD80 and secrete cytokines as shown by stimulation via TLR4 with LPS (Fig. 3).

To investigate whether primary CTL responses were induced in vivo by CpG-OVA conjugates, TLR9-deficient and wt mice were challenged s.c. in the footpad with immunostimulatory CpG-OVA conjugates or OVA. After 4 days, cells of the draining LN s were harvested, cultured for an additional 72 h, and tested for CTL activity. As shown in Fig. 4, wt but not TLR9-deficient mice generated SIINFEKL-specific CTL in response to CpG-OVA conjugates. However, Ag-specific CTL were generated in both TLR9<sup>−/−</sup> and wt mice when OVA was administered i.p.

In conclusion, these data provide compelling evidence that, following administration of CpG-Ag conjugates, the function of TLR9 is restricted to activation of OVA cross-presenting DCs in professional APCs. CpG DNA-activated cellular uptake and cross-presentation of linked OVA does not involve TLR9. The enhanced Ag loading of DC with OVA seen by conjugation with CpG DNA (9, 17) (18) must be mediated by a yet-undefined DNA-binding receptor lacking nucleic acid sequence specificity. Following endocytosis, immunostimulatory CpG DNA meets TLR9 in late endosomes where the adaptor molecule MyD88 is recruited (13, 19). This contrasts with LPS, which activates TLR4 expressed at the cell membrane (13, 20). Of note, cross-presentation of conjugated Ag by TLR9<sup>−/−</sup> DCs is qualitatively similar to the pattern seen in wt DCs independently of the CpG sequence used (9). In contrast, activation of cross-presenting DC to allow cross-priming requires the presence of TLR9 and immunostimulatory CpG DNA (Fig. 3) (9). Thus, the use of DNA-OVA conjugates provides opportunities to influence the subsequent adaptive immune response, first by enhancement of Ag uptake, and second by DC activation through TLR9. Future studies can address the question of whether CpG-Ag conjugates are primarily cross-presented by CD8<sup>+</sup> DCs (3, 21) and whether CpG-Ag conjugates in TLR9<sup>−/−</sup> mice or nonstimulatory DNA-Ag conjugates in wt mice (9) efficiently induce cross-tolerance.

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References


