CpG-DNA aided cross-presentation of soluble antigens by dendritic cells

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For cross-presentation immature dendritic cells (DC) require enhanced antigen (Ag) uptake and a maturation signal to prime for MHC class I-restricted CTL responses *in vivo*. While immunostimulatory CpG-DNA provides, via TLR9, the maturation signal, CpG-DNA linked to Ag augments cellular Ag uptake. In this study we show that CpG-DNA ovalbumin (OVA) conjugates trigger *in vivo* peptide-specific CTL responses at tenfold lower Ag doses compared to a mixture of CpG-DNA plus OVA. We provide evidence that CpG-DNA-OVA conjugates shift OVA uptake by immature DC from the presumably inefficient fluid phase pinocytosis to efficient DNA receptor-mediated endocytosis. Since the DNA-binding receptor mediating endocytosis lacks any sequence specificity, cellular uptake of OVA conjugated with either stimulatory or non-stimulatory oligonucleotides (ODN) is equally enhanced. As a consequence cross-linking of OVA with either stimulatory or non-stimulatory DNA yields, via enhanced OVA uptake, efficient generation and presentation of the dominant OVA-CTL epitope SIINFEKL. However, only stimulatory CpG-ODN cross-linked to OVA provide the DC maturation signal required to trigger robust primary CTL responses towards the cross-presented MHC class I complexed T cell epitope SIINFEKL. Our studies show that stimulatory CpG-ODN linked to Ag fulfill a dual role: enhancement of Ag uptake yielding efficient Ag cross-presentation by DC and in addition, their activation into professional DC.

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1 Introduction

Dendritic cells (DC) are crucial for the initiation of primary T cell responses [1]. Immature DC lack costimulatory signals required for productive T cell activation but are well equipped to sample Ag, for example, via receptor-mediated endocytosis or fluid phase pinocytosis [2, 3]. DC maturation is efficiently brought about, for example, by bacteria-derived ligands of the toll-like receptor (TLR) family members [4, 5] or CD4 T helper (Th)-dependent CD40 ligation [6, 7]. Following DC maturation Ag sampling ceases, expression of costimulatory molecules and MHC-peptide complexes increases [8, 9] and cytokines including the Th1 and CTL promoting IL-12 are produced [1].

In general, MHC class I molecules are complexed with peptides (CD8 T cell epitopes) derived from cytosolic Ag.

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The first two authors contributed equally to this work.

Abbreviation: **TLR:** Toll-like receptor

However, when DC internalize high amounts of exogeneous Ag they appear to be able to route internalized Ag into the class I MHC presentation pathway [10, 11], a phenomenon originally termed "cross-presentation" [12, 13]. A bottle neck for internalization of soluble exogeneous Ag by immature DC represents the low efficacy of fluid phase pinocytosis, presumably because it lacks selectivity for the antigen in question. The efficacy of antigen internalization, however, is greatly enhanced when receptor-mediated endocytosis comes into function, examples being C-type lectins that bear mannose specificity [14], Ag/Ab immune complexes that bind the FcγR on DC [15] or Ag delivered via apoptotic cells [16]. Interestingly, these receptor-dependent internalization routes are associated with efficient Ag cross-presentation [14, 15].

Immunostimulatory bacterial CpG-DNA [17] activates immature DC via TLR9 [18–20]. To accomplish this, CpG-DNA needs first to be taken up by DC via receptor-mediated endocytosis [21, 22], since CpG-DNA activates via TLR9 the Toll/IL-1R signal pathway at late

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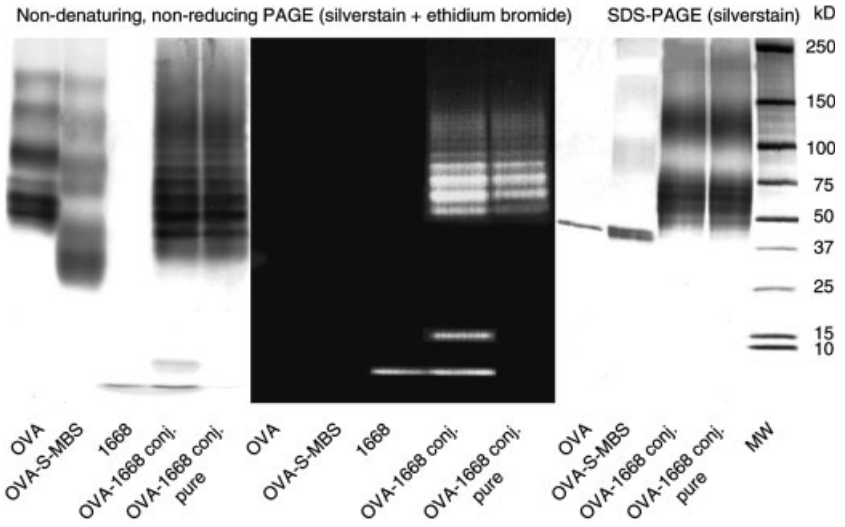

Fig. 1. Gel analysis of conjugates using a non-denaturing, non-reducing 4–15% PAGE or a 6–20% gradient SDS-PAGE which were consecutively silver stained or visualized under short-wave UV-light; the ethidium bromide stain selectively visualizes ODN but not OVA by itself. After purification by chromatography the conjugates are free from unbound ODN monomers or dimers. Shown by SDS-PAGE OVA (~45 kDa) and the shifting of the OVA-band after having bound ODN (~6.5 kDa) can be observed. The molar ratio of bound ODN per molecule OVA is approximately 2.5:1.

(Lamp1$^+$) endosomal organelles [22, 23]. Interestingly, challenge of mice with a mixture of CpG-DNA (used as adjuvants) and rather high concentrations of soluble antigen [ovalbumin (OVA), β-galactosidase] triggers peptide-specific CTL in the draining lymph nodes (LN) [24], even in the absence of Th cells [25]. Others [26, 27] have reported that if CpG-DNA is covalently linked to Ag, cellular uptake of Ag by DC is enhanced, a phenomenon poorly understood.

Here, we explored the immunobiology of CpG-DNA-conjugated soluble Ag, both *in vitro* and *in vivo*. We provide evidence that conjugated DNA sequence nonspecifically triggers receptor-mediated endocytosis of Ag,

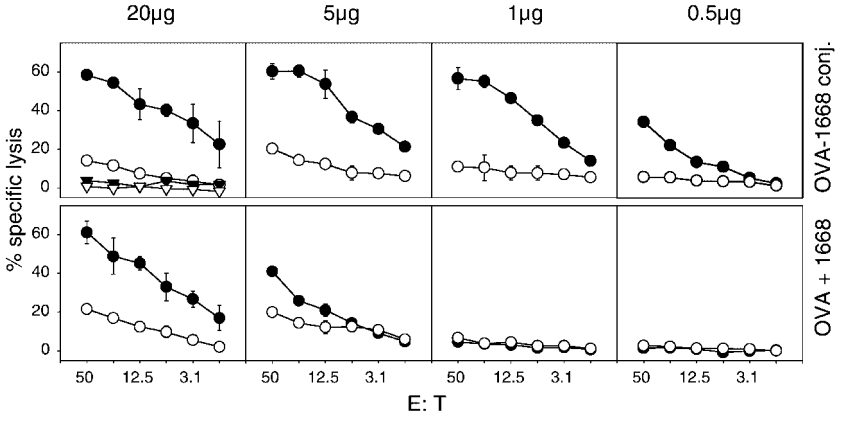

Fig. 2. Enhanced immunogenicity of OVA-CpG conjugates. C57BL/6 mice were challenged into hind footpaths with OVA mixed or conjugated with the CpG-DNA 1668 at protein amounts ranging from 5 to 0.5 µg (equaling 280 to 28 pmol ODN). After 4 days draining LN were harvested, cells were isolated and cultured for additional 4 days before a chromium-release assay was performed. Cultured LN cells from mice immunized with 50 µg OVA conjugated to the control GpC-DNA 1720 (2.8 nmol ODN; triangles) as well as LN cells from mice immunized with OVA only (not shown) did not elicit CTL. Solid symbols: EL-4 target cells pulsed with the OVA peptide SIINFEKL; open symbols: unpulsed EL-4 cells. A representative experiment (out of three) is shown.

which in turn becomes processed to MHC class I epitopes. In addition, once the CpG-Ag conjugate has been ingested, CpG-DNA sequence specifically triggers maturation of DC loaded with antigen. As a consequence, soluble antigen becomes efficiently cross-presented by activated DC that drive robust CTL responses *in vivo*.

2 Results

2.1 Protein CpG-DNA conjugates induce robust CTL responses

We selected OVA as antigen, since its MHC class I-restricted CD8 T cell epitope SIINFEKL is well characterized. OVA was chemically conjugated to either the immunostimulatory CpG-oligonucleotide (ODN) 1668 or the non-stimulatory GpC-ODN 1720. The PAGE analysis of purified OVA-CpG conjugates is shown in Fig. 1. Typically an average of 2.5 CpG-ODN were conjugated per molecule OVA.

In the first set of experiments the ability of OVA-CpG conjugates to induce primary CTL responses *in vivo* was compared to that of a mixture of CpG-DNA plus OVA. Since a mixture of CpG-DNA plus OVA requires 20–50 μg OVA for CTL induction [24, 25] groups of mice were challenged s.c. with graded concentrations of OVA plus CpG-DNA (mixture) or of CpG-OVA conjugates, respectively. Fig. 2 details a typical experiment to show that s.c. challenge with 5 μg OVA (mixed with CpG-DNA) was the lowest Ag concentration that triggered peptide-specific CTL in draining LN. In contrast, tenfold less OVA in form of CpG-OVA conjugate was required to induce primary CTL responses similar in magnitude, provided it contained stimulatory CpG-DNA. Non-stimulatory GpC-OVA conjugates failed to induce CTL at all concentrations tested. We concluded, that OVA conjugated with stimulatory CpG-ODN is about tenfold more immunogenic compared to a mixture of OVA plus CpG-DNA, non-stimulatory GpC-OVA complexes being inactive. We also analyzed whether immunogenic CpG-OVA conjugate would display side effects observed with "free" CpG-DNA, that is induction of systemic but transient levels of proinflammatory cytokines [28]. As shown in Table 1, only marginal concentrations of TNF-α and IL-12 p40 was found in the serum of mice challenged with CpG-OVA conjugates.

Table 1. Serum cytokine levels 2 h after i.p. injection[a)]

Antigen used for immunization	IL-12p40 (pg/ml)	TNF-α (pg/ml)
OVA	8.1±1	13.7±2.1
OVA + 1668	1,443±37	233±48
OVA – 1668 conj.	48.6±6	48.4±30.6

a) Serum cytokine levels in mice injected with OVA-CpG conjugates or OVA plus CpG-ODN mixtures. Three mice per group were injected i.p. with 5 nmol/mouse 1668 CpG-ODN either mixed or conjugated with 107.5 μg OVA. One mouse injected with 107.5 μg OVA in PBS served as control. Two hours later serum of mice was collected. Serum concentrations of cytokines (IL-12 p40 and TNF-α) were determined in triplicates by commercially available ELISA kits. Mean and standard deviation is shown.

2.2 Enhanced *in vitro* and *in vivo* uptake of stimulatory and non-stimulatory DNA-OVA complexes parallels enhanced SIINFEKL presentation

Stimulatory CpG-DNA needs to be taken up, via receptor-mediated endocytosis, in order to activate TLR9 in the late endosomal compartment [21–23]. To analyze the mechanisms of uptake we conjugated CpG-DNA to FITC-labeled OVA, exposed bone marrow-derived DC to these reagents, and analyzed cellular uptake by flow cytometry. Fig. 3 details a typical experiment with Flt3-ligand-cultured $CD11c^+$ DC exposed to either OVA-FITC (0.5 μg/ml) or OVA-FITC (0.5 μg/ml) mixed with CpG-DNA yielding about 5–7% FITC-positive DC, while CpG-conjugated OVA-FITC (0.5 μg) was taken up by ca. 32% of DC. To quantify the efficacy of uptake, DC were additionally exposed to graded dilutions of CpG-OVA-FITC conjugates, and analyzed in parallel. As shown (Fig. 3), uptake of CpG-conjugated OVA equaled that of OVA-FITC at about 20-fold lower concentration. To exclude mere surface binding treated DC in addition were cytospun on glass slides. When examined by confocal microscopy intracellular compartimentation of OVA-FITC was verified (data not given). Of note, cellular uptake of OVA conjugated with non-stimulatory GpC-DNA was equally enhanced (see below). Since immature DC constitutively process proteinaceous Ag to MHC-restricted peptides [11], we predicted that "loading" of DC with OVA conjugated either with stimulatory CpG-DNA (1668) or non-stimulatory GpC-DNA (1720) should be equally effective in generating MHC class I molecules complexed with SIINFEKL peptides. To test this prediction we used as "read-out" the activation of SIINFEKL-specific B3Z hybridoma cells. As shown in Fig. 4, DC "loaded" with OVA conjugated either with stimulatory CpG-DNA or non-stimulatory GpC-DNA generated and presented equally well MHC class I complexed SIINFEKL. In contrast, the efficacy of OVA or a mixture of OVA plus CpG-DNA to be cross-presented was poor.

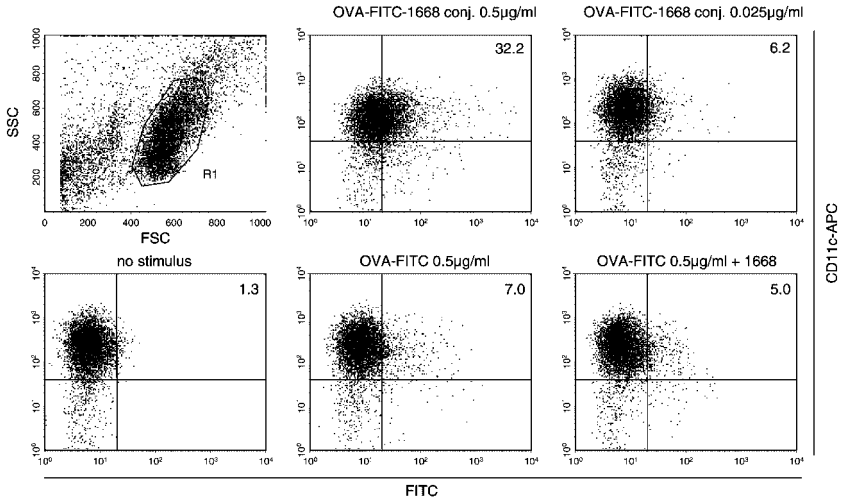

Fig. 3. Enhanced uptake of OVA-CpG-DNA conjugates in primary DC. Flt3-ligand cultured bone marrow-derived DC were incubated with 0.5 or 0.025 µg/ml of OVA-FITC alone, mixed or conjugated with the CpG-DNA 1668 (22.5 nM or lower) for 90 min at 37°C, stained with APC-labeled anti-CD11c and analyzed by FACS. Percentages of FITC-positive, Ag-bearing $CD11c^+$ DC are shown.

The results in Fig. 4 imply that DNA aided "loading" of DC with conjugated OVA is sequence nonspecific. If the DNA-binding receptor mediating endocytosis lacked any sequence specificity, uptake of CpG-DNA-conjugated OVA by DC ought to be competed for by excess of "third party" ODN. The results detailed in Fig. 5 confirm this prediction since "third party" ODN dose dependently inhibited uptake of OVA conjugated with CpG-DNA. To ensure that cellular uptake was inhibited, trypan blue was used to quench surface-bound FITC in these experi-

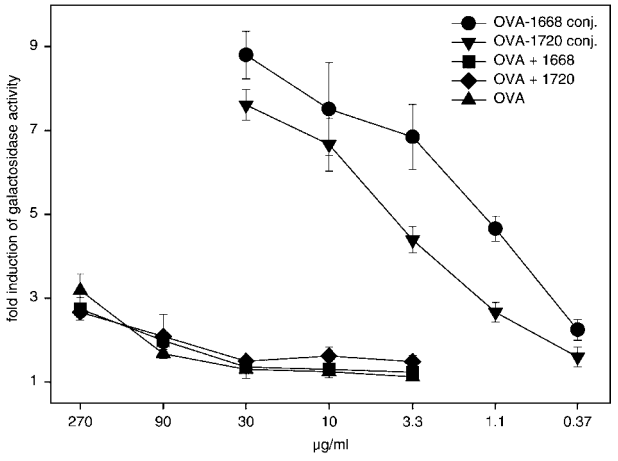

Fig. 4. Sequence-nonspecific enhancement of SIINFEKL presentation by OVA-DNA conjugates. DC from Flt3-ligand conditioned bone marrow cell cultures were incubated in triplicates with OVA alone, mixed or conjugated with either the CpG-DNA 1668 or the GpC-DNA 1720 for 5 h. Cells were washed and incubated overnight with B3Z cells and induced β-galactosidase activity was measured. Fold induction of β-galactosidase activity compared to DC incubated with no stimulus is shown. Amount of protein (µg/ml) used is indicated.

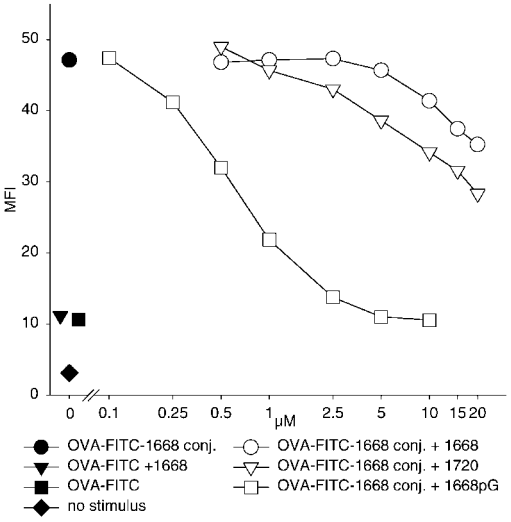

Fig. 5. "Third party" DNA block uptake of FITC-labeled OVA-CpG-DNA conjugates. ANA-1 were incubated for 1 h at 37°C with either 4 µg/ml of OVA-FITC alone, mixed or conjugated with the CpG-ODN 1668 (180 nM) or no stimulus (solid symbols). Increasing amounts of free CpG-ODN (1668), GpC-ODN (1720) or CpG-ODN modified with a polyguanosine tail (1668pG) were added to cultures containing FITC-labeled OVA-CpG-DNA conjugates (open symbols). OVA-FITC uptake was analyzed by FACS. To ensure intracellular uptake potential OVA-FITC surface staining was quenched by adding 50 µg/ml trypan blue. Mean fluorescence intensity of the FITC-signal is shown.

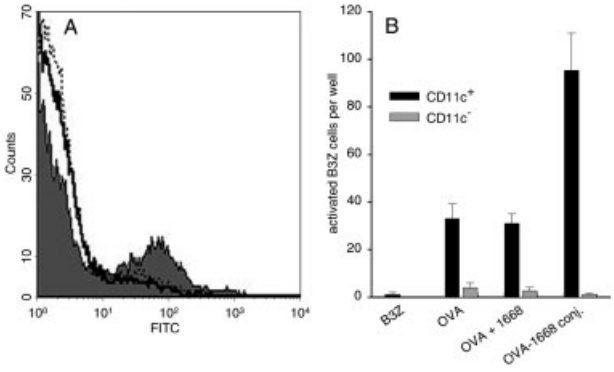

Fig. 6. (A) *In vivo* uptake of FITC-labeled OVA-CpG-DNA conjugates by DC. To examine uptake of FITC-labeled conjugates *in vivo*, mice were injected s.c. with 0.5 μg of OVA-FITC conjugated with 2.8 pmol 1668 CpG-DNA (shadowed area) or a mixture of 0.5 μg OVA-FITC plus 2.8 pmol 1668 CpG-DNA (thick lines) or 0.5 μg OVA-FITC in PBS (dotted line) per footpad. After 4 h, draining LN were removed, digested for 1 h at 37°C using collagenase Type Ia and single-cell suspensions were prepared. After separation in $CD11c^+$ and $CD11c^-$ cell fractions, cells were stained with anti-CD11c-APC and analyzed by FACS. Only $CD11c^+$ cells are shown. (B) SIINFEKL presentation by OVA-DNA conjugates *in vivo*: To evaluate SIINFEKL presentation, DC were treated similarly with unlabeled OVA as in (A) and subsequently incubated with 10^6 SIINFEKL/K^b-specific B3Z cells. After additional treatment with X-Gal-solution for 4–8 h, activated "blue" B3Z cells were counted by microscopy. B3Z incubated with medium only served as control. Mean and standard deviation of three wells are shown.

ments. Similar results were obtained with bone marrow-derived DC (data not shown). We concluded that CpG-DNA conjugated to OVA aids endocytosis of OVA, by routing the CpG-OVA complex to the receptor-mediated endocytosis pathway known to internalize sequence nonspecifically CpG-DNA [21].

To verify aspects of these data under *in vivo* conditions, DC from draining LN were positively selected via $CD11c^+$ beads 4 h after local s.c. challenge with either OVA-FITC, a mixture of OVA-FITC plus CpG-DNA or CpG-OVA conjugates and subsequently analyzed for their OVA-FITC content by flow cytometry. In this *in vivo* setting uptake of CpG-OVA-FITC conjugates was clearly enhanced (Fig. 6A). Furthermore CpG-DNA aided uptake of OVA resulted in enhanced presentation of the MHC class I-restricted SIINFEKL (Fig. 6B).

2.3 DC activation by CpG-OVA conjugates

Next we addressed the question whether upon receptor-mediated endocytosis stimulatory CpG-DNA covalently conjugated to OVA was still able to activate DC. To this bone marrow-derived immature DC were exposed to OVA conjugated with stimulatory CpG-DNA, a mixture of OVA plus CpG-DNA or OVA alone. As shown in Fig. 7 CpG covalently linked to OVA triggered up-regulation of the costimulatory molecules CD40 and CD86 almost as efficiently as unconjugated CpG-DNA. However, the ability to induce cytokines was somehow dampened. For example, production of IL-6 was significantly reduced and biologically active IL-12 p70 required higher doses of CpG-OVA complexes (data not shown).

3 Discussion

This study describes that administration of Ag to immature DC in the form of CpG-Ag conjugates endows

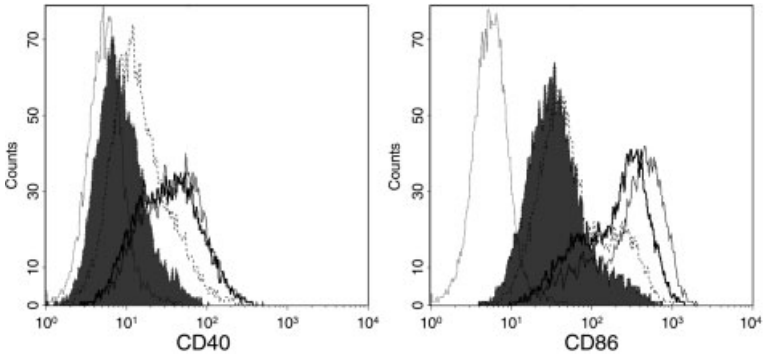

Fig. 7. Costimulatory molecule expression on primary DC is increased by CpG-DNA conjugated OVA. Flt3-ligand-cultured DC were incubated for 16 h at 37°C with 17.6 μg/ml of OVA alone, mixed or conjugated with 1 μM CpG-DNA (1668). Cells were washed and stained for CD11c, CD40 and CD86 expression. Only live and $CD11c^+$ cells are shown. Thick line: OVA-1668 conjugate; thin line: OVA + 1668; broken line: OVA; shadowed area: no stimulus; dotted: isotype.

DC with the capacity to cross-present MHC class I restricted T cell epitopes that trigger *in vivo* primary CTL responses. At least two mechanisms contribute to this phenomenon. First, upon conjugation with CpG-DNA uptake (endocytosis) of Ag is greatly improved, leading to enhanced presentation of processed MHC class I-restricted peptides. Secondly, upon endocytosis of the CpG-Ag complexes linked CpG-DNA is still able to activate DC.

Bacterial CpG-DNA acts as pathogen-associated molecular pattern (PAMP) that is sequence specifically recognized by the pattern recognition receptor TLR9 [19, 20]. Using TLR9 specific mAb we described that murine innate immune cells express TLR9 cytoplasmatically at endosome-like organelles, but not at the cell membrane. As a consequence CpG-DNA needs to be endocytosed to meet TLR9 at the endosomal compartment [23]. Upon endosomal maturation (acidification) CpG-DNA recruits, via TLR9, the adaptor molecule MyD88 thus initiating signaling via the Toll/IL-1R signal pathway [21, 23].

Given this information, it is not surprising that cellular uptake (endocytosis) of CpG-DNA represents a prerequisite for recognition by TLR9. Uptake of CpG-DNA via endocytosis is receptor-mediated for the following reasons: first, cellular uptake is competed for by "third party" DNA, particularly effective by those rich in G-sequences [21, 29]. This implies involvement of a cell surface DNA-binding receptor lacking sequence specificity. Secondly, the endocytosis inhibitor Monodansylcadaverin [30] effectively blocks uptake of stimulatory CpG-DNA [23], and thus initiation of signaling. Thirdly, as shown here, conjugation of either stimulatory or non-stimulatory CpG-ODN to OVA effectively augmented cellular uptake (loading) of OVA. Thus we conclude that cellular uptake of ODN requires a receptor that lacks sequence specificity. Furthermore the data imply that it is DNA conjugated to OVA that targets OVA to the DNA-binding receptors on DC to bring about receptor-mediated endocytosis. As a consequence, cellular uptake of OVA shifts from inefficient fluid phase pinocytosis [2] to efficient receptor-mediated endocytosis [14]. DC loaded via receptor-mediated endocytosis with high concentrations of Ag are able to route their cargo into the MHC class I presentation pathway. Even if the results in Fig. 3 numerically suggest modest (fivefold) enhanced uptake of CpG conjugated OVA, it is noteworthy that the whole DC population has shifted (Fig. 3). Furthermore because OVA is highly mannosylated it could interact with other endocytotic receptors, thus explaining the background (5–6% of DC take up FITC-conjugated OVA). Enhancement of Ag uptake via receptor-mediated endocytosis is precedential. For example, presentation of exogeneous Ag via MHC class I can be improved by complexing Ag to beads [31], by providing Ag in bacteria [32] or as Ag-Ab immune complexes thereby accessing the FcγR-mediated endocytosis pathway [33] or by administering Ag in apoptotic cells [16]. Interestingly, a common denominator of these various approaches to augment Ag uptake by DC is the ability of the "loaded" DC to cross-present soluble Ag.

To induce productive T cell responses peptide-presenting immature DC need to transit to professional DC [7]. Here we show that upon "loading" of DC with stimulatory CpG-conjugated OVA, the "loaded" DC increase expression of costimulatory CD40 and CD86 molecules, and produce cytokines, such as IL-6. It follows that upon endocytosis stimulatory CpG-DNA linked to OVA either becomes liberated by enzymatic activities within the mature endosome, or is able to activate TLR9 in its linked form. When conjugated to OVA the efficacy of CpG-DNA-mediated DC activation appeared to be reduced, since we could detect only IL-12 p70 production *in vitro* if higher concentrations of OVA-CpG-DNA conjugates were used.

Cross-linking of immunostimulatory DNA sequences with proteinaceous Ag results in CTL priming and Th1-biased immune responses as first reported by Raz and colleagues [34]. In murine bronchial asthma models CpG-Ag conjugates were superior in regulating murine airway eosinophilia and Th2 cells as compared to mixtures of Ab plus CpG-DNA [26, 35]. Using phycobiliprotein-CpG-DNA conjugates Shirota et al. [26] were first to document DNA-guided augmentation of Ag sampling by DC. Building on these observations, we compared here the efficacy of CpG-OVA conjugates, of GpC-OVA conjugates and of unconjugated mixtures to cross-present OVA for primary peptide-specific CTL responses. We conclude that CpG-OVA conjugates shift OVA uptake by DC from the rather inefficient fluid phase pinocytosis to efficient receptor-mediated endocytosis. Concomitantly the OVA-loaded immature DC process the MHC class I-restricted peptide SIINFEKL. Since the DNA-binding receptor mediating endocytosis lacks any sequence specificity, stimulatory as well as non-stimulatory ODN linked to OVA guide augmented OVA uptake. However, only the former cause activation of SIINFEKL-presenting immature DC into mature professional APC while the latter lacks this ability. We are aware that identification of the DNA-binding receptor mediating cellular uptake of linked Ag is operational as yet. Therefore we currently investigate the nature of the receptor that initiates sequence nonspecifically endocytosis of ODN-OVA conjugates in DC.

4 Materials and methods

4.1 Mice

C57BL/6 mice were purchased from Harlan Winkelmann GmbH, Germany. All animals were kept under specific pathogen-free conditions and were used at 8–12 weeks of age.

4.2 Cell lines and *in vitro* culture medium

EL-4 (H-2^b) thymoma cells and ANA-1 (H-2^b), a macrophage cell-line from C57BL/6 mice, were purchased from the American Type Culture Collection (Rockville, MD). B3Z, a somatic T cell hybrid generated by fusing the OVA/K^b-specific cytotoxic clone, B3, with a lacZ-inducible derivative of BW5147 fusion partner [36], was kindly provided by Dr. B. L. Kelsall (National Institutes of Health, Bethesda, MD). Cells were cultured in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 IU/ml penicillin G, 100 IU/ml streptomycin sulfate (all: Biochrom KG, Germany) and 50 μmol/l 2-mercaptoethanol (Gibco BRL Lifetechnologies GmbH, Germany) at 37°C/5% CO_2.

4.3 Generation of Flt3-ligand-cultured DC from bone marrow

Flt3-ligand supplemented bone marrow cell cultures were generated as described with slight modification [37]. Briefly, bone marrow cells were flushed out of femurs and tibiae, centrifuged and red blood cells were lysed for 2 min with tris-ammonium chloride at 37°C. After washing, cells were plated at 1.5×10^6 cells/ml in media complemented with 200 ng/ml hu Flt3-ligand (Amersham Biosciences, St Louis, MO) and cultured at 37°C/5%CO_2. Cells were used after 10 days of culture.

4.4 Reagents and monoclonal antibodies

PBS was purchased from PAN Biotech, Germany. Chicken egg albumin (OVA) was from Sigma, Germany. The peptide SIINFEKL (OVA peptide 257–264) was custom-synthesized by Research Genetics Inc. (Huntsville, AL). FITC-labeled OVA was purchased from Molecular Probes, Leiden, The Netherlands. Phosphothioate-modified ODN were custom-synthesized by MWG, Germany. The sequences used were: 1668: 5'-TCC AT**G A<u>CG</u> TT**C CTG ATG CT-3'; 1720: 5'-TCC AT**G A<u>GC</u> TT**C CTG ATG CT-3'; 1668pG: 5'-TCC AT**G A<u>CG</u> TT**C CTG GGG GG-3' (the stimulatory CpG- and the non-stimulatory CpC-motifs with flanking nucleotides are highlighted; see also Sect. 4.5). APC-labeled anti-CD11c (clone HL3), FITC-labeled anti-CD40 (clone 3/23), FITC-labeled anti-CD86 (clone GL1) mAb and corresponding isotype controls were purchased from PharMingen, Germany.

4.5 Conjugation of ODN to protein and analysis of conjugates

The phosphothioated sulfhydryl-modified ODN (TriLink Biotechnologies, La Jolla, CA) used throughout this study consisted of 20 bases and contained either a CpG motif (1668: 5'-S-TCCAT**GA<u>CG</u>TT**CCTGATGCT-3') or the corresponding GpC control motif (1720: 5'-S-TCCAT**GA<u>GC</u>TT**CCTG-ATGCT-3'). Ovalbumin was incubated with the cross-linker sulfo-maleimidobenzoyl-N-hydroxysuccinimide ester (S-MBS; Pierce, 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-dithiothreitol-PBS solution. Subsequently unbound S-MBS and 1,4-dithiothreitol were removed by chromatography on a Bio-Rade P-2 gel column (Bio-Rad, 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 L-cysteine was added to quench reactive S-MBS. Free ODN were removed by chromatography on a Superdex 75HR column (Amersham Biosciences, Germany). Purified conjugates were analyzed on a 6–20% gradient SDS-PAGE and consecutively silver stained. To determine ratio of bound ODN on OVA a 4–15% gradient non-denaturing, non-reducing PAGE was run and silver stained or visualized using ethidium bromide staining. Protein concentration was determined by the Lowry method (Pierce, Germany).

4.6 Immunization and chromium-release assay

For induction of CTL, protein and adjuvant were injected into both hind footpads of mice. Four days later draining LN were removed and a single-cell suspension was prepared. LN cells (3×10^6 cells/ml) were cultured for additional 4 days in media conditioned with 10 IU/ml rIL-2. The ^{51}Cr-release assay was performed as described [38]. In short, 2×10^6 EL-4 target cells were labeled with 150 μCi $Na_2{}^{51}CrO_4$ (Amersham Biosciences, Germany) for 1 h at 37 °C. Half of the cells were subsequently incubated with peptide solution (0.1 μM of SIINFEKL) for additional 30 min. Peptide-untreated cells served for specificity control. After washing 10^3 target cells were incubated with replicate serial dilutions of CTL at 37°C/5%CO_2. After 4-h culture supernatant from each well was used to detect gamma irradiation. Specific lysis was calculated according to the formula: % specific lysis = [cpm(sample) – cpm(spontaneous release)/(maximum release) – cpm(spontaneous release)]×100.

4.7 Uptake and activation analysis

To examine the uptake of FITC-labeled conjugate *in vivo* 0.5 μg protein (2.8 pmol ODN) was injected s.c. into the footpads of mice. LN were aseptically removed and digested for 1 h at 37°C/5%CO_2 using collagenase type Ia (Sigma, Germany). Single-cell suspensions were prepared and clumps removed using a 100-μm pore size filter (Falcon,

Germany). Thereafter cells were stained with magnetic beads coated with CD11c mAb and separated in $CD11c^+$ and $CD11c^-$ cell fractions using MiniMACS and MS^+ separation columns according to the manufacturer's protocol (Miltenyi Biotec, Germany).

To examine the uptake of FITC-labeled conjugates *in vitro* bone marrow-derived DC were exposed to FITC-labeled Ag (1 h at 37°C), washed with ice-cold 2% FCS-PBS containing 2 mM EDTA and stained with APC-labeled anti-CD11c. To examine the ability of "third party" ODN to block uptake of FITC-labeled conjugates, cells of the macrophage line ANA-1 or immature DC were incubated with OVA-FITC alone, mixed or conjugated with the CpG-ODN 1668 (180 nM) for 1 h at 37°C. Increasing concentrations of free CpG-ODN (1668), GpC-ODN (1720) or CpG-ODN modified with a polyguanosine tail (1668pG) were added. To ensure intracellular uptake, surface staining of OVA-FITC was quenched by adding 50 μg/ml of trypan blue [39].

To analyze activation of DC, Flt3-ligand-cultured bone marrow-derived DC were incubated with 17.6 μg/ml of OVA alone, mixed or conjugated to 1 μM CpG-DNA. Cells were cultured for 24 h, thereafter washed and stained with APC-labeled anti-CD11c, FITC-labeled anti-CD40 or FITC-labeled anti-CD86. FACS analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, Germany) acquiring at least 30,000 events per sample. FACS data were analyzed using CellQuest software.

4.8 Presentation assay

Presentation of SIINFEKL *ex vivo* was assayed as previously described [38] by measuring induction of lacZ activity in the SIINFEKL/K^b-specific T cell hybridoma B3Z [36]. To this B3Z cells and positively selected $CD11c^+$ LN cells were cocultured. Briefly, 12 h after Ag injection the draining LN were harvested and dissociated LN cells were exposed to magnetic beads coated with anti-CD11c mAb. For separation into $CD11c^+$ and $CD11c^-$ subpopulations MiniMACS and MS^+ separation columns according to manufacturer's instructions were used (Miltenyi Biotech, Germany). Defined numbers of fractionated cells were incubated with B3Z at 37°C overnight. Thereafter cells were fixed with 0.5% glutaraldehyd for 10 min and incubated with X-Gal-Solution [40] at 37°C. After 4–8 h "blue" B3Z cells were counted under the microscope.

To evaluate the SIINFEKL presentation *in vitro* 2×10^5 Flt3-ligand-cultured cells were incubated with indicated substances for 5 h at 37°C. Plates were washed and 5×10^4 B3Z cells were added to each well. After additional incubation overnight at 37°C cells were lysed by addition of 100 μl Z-buffer (100 mM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% Nonidet P-40, 0.15 mM chlorophenol red β-galactoside (Calbiochem, San Diego, CA) in PBS) and after 24 h absorption of individual wells was read using a 96-well Emax plate reader (Molecular Devices, Sunnyvale, CA) at 570 nm, with 650 nm as reference wave length.

4.9 Detection of cytokines

For the serum determination of cytokines 5 nmol/mouse CpG-DNA conjugated or mixed with OVA (107.5 μg) was injected i.p. Two hours later serum of mice was collected. Cytokine release (IL-12p40, TNF-α) was determined in triplicates by ELISA kits (duo set for IL-12p40 and TNF-α, R&D Systems, Germany). Assays were performed as described by the manufacturer.

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