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J Immunol 2000; 164:2372-2378; doi: 10.4049/jimmunol.164.5.2372
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CpG-DNA Activates In Vivo T Cell Epitope Presenting Dendritic Cells to Trigger Protective Antiviral Cytotoxic T Cell Responses

Ramunas M. Vabulas,*† Hanspeter Pircher,‡ Grayson B. Lipford,* Hans Häcker,* and Hermann Wagner‡*

MHC class I-restricted T cell epitopes lack immunogenicity unless aided by IFA or CFA. In an attempt to circumvent the known inflammatory side effects of IFA and CFA, we analyzed the ability of immunostimulatory CpG-DNA to act as an adjuvant for MHC class I-restricted peptide epitopes. Using the immunodominant CD8 T cell epitopes, SIINFEKL from OVA or KAVYNFATM (gp33) from lymphocytic choriomeningitis virus glycoprotein, we observed that CpG-DNA conveyed immunogenicity to these epitopes leading to primary induction of peptide-specific CTL. Furthermore, vaccination with the lymphocytic choriomeningitis virus gp33 peptide triggered not only CTL but also protective antiviral defense. We also showed that MHC class I-restricted peptides are constitutively presented by immature dendritic cells (DC) within the draining lymph nodes but failed to induce CTL responses. The use of CpG-DNA as an adjuvant, however, initiated peptide presenting immature DC progression to professional licensed APC. Activated DC induced cytolytic CD8 T cells in wild-type mice and also mice deficient of Th cells or CD40 ligand. CpG-DNA thus incites CTL responses toward MHC class I-restricted T cell epitopes in a Th cell-independent manner. Overall, these results provide new insights into CpG-DNA-mediated adjuvanticity and may influence future vaccination strategies for infectious and perhaps tumor diseases. The Journal of Immunology, 2000, 164: 2372–2378.

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Received for publication September 13, 1999. Accepted for publication December 17, 1999.

Cytotoxic CD8 T lymphocytes (CTL) recognize antigenic peptides presented by MHC class I molecules, whereas CD4 Th cells detect peptides presented by MHC class II (1). CTL priming usually requires active involvement of Th cells (2–4). T cell help is principally mediated indirectly by Th cells or Th den-

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allows Th cell-independent CTL responses to MHC class I-restricted T cell epitopes.

**Materials and Methods**

**Reagents**

t-L-Phosphatidylcholine (type XI-E), t-phosphatidyl-cytidol, cholesteryl, and IFA were purchased from Sigma (Munich, Germany). OVA peptide 257–264 (SIINFEKL) and LCMV glycoprotein 33–41 peptide (KAVYNFATM) were custom synthesized by Research Genetics (Huntsville, AL). For technical reasons (to prevent dimer formation), the original cysteine at the anchor position 41 in the gp33 had been replaced by methionine. Phosphorothioate-stabilized oligodeoxynucleotides (ODN) were synthesized by TibMolBio! (Berlin, Germany). CpG-ODN (ODN 1668, containing a CG-motif marked with bold letters: 5′-TCC-ATG-ACG-CTG-CTG-ATG-CTG-CTG-CTG-CTG) and control GpC-ODN (inverted CG = ODN 1720: 5′-TCC-ATG-ACG-CTG-CTG-CTG-CTG-CTG-CTG) were taken from Ref. 24.

**Mice**

Female C57BL/6 J mice were purchased from Harlan Winkelmann (Borchen, Germany). CD40L knockout mice (C57BL/6J-Tnfsf5tm1Imx) were kindly provided by Dr. I. Förster (Technical University Munich, Munich, Germany). All animals were housed 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 American Type Culture Collection (Rockville, MD). B2Z, a somatic T cell hybrid generated by fusing the OVA/Kb-specific cytotoxic clone B3, with a La2-inducible derivative of BW5147 fusion partner (26), was kindly provided by Dr. B. L. Kelsall (National Institutes of Health, Bethesda, MD). Cells were cultured in Clinks/RPMI (Biochrom, Berlin, Germany) supplemented with 10% (v/v) heat-inactivated FCS (Biochrom), 5 mM 2-ME, and antibiotics (100 IU/ml penicillin G and 100 IU/ml streptomycin sulfate) at 37°C and 5% CO2.

**Peptide-liposome formation**

Liposomes were manufactured by a rehydration entrapment method (27). Briefly, 18 mg phosphatidylcholine, 2 mg phosphatidylglycerol, and 5 mg cholesterol (2:0.2:1) were suspended in 5 ml chloroform in a 250-ml round bottom flask. The mixture was rotary evaporated under reduced pressure until a thin lipid film formed on the flask wall. Residual chloroform was removed by vacuum desiccation. The peptide (3 mg) was solubilized in a minimal volume of water and diluted to 1 ml with PBS. This solution was slowly added to the dried lipid and hand shaken until the lipids were resuspended. The mixture was then filter extruded through a 0.2-μm pore size Anotop 10 Plus syringe mount filter (Whatman, Maidstone, England).

**Immunization and 51Cr release assay**

For priming of cytotoxic T cells, 0.3 μg of respective peptide entrapped in liposomes or solubilized in PBS were injected into both fore footpads of mice. As an adjuvant CpG-ODN was cojected (10 nmol/mouse). After 4–5 days, draining LN were removed and a single-cell suspension was prepared by pressing the LN through a screen. LN cells were cultured at 37°C for 4 days in a virus plaque assay, as described previously (28). LCMV was harvested from each well and titered in the spleens were determined after 4 days in a virus plaque assay, as described previously (28). LCMV was quantified with an immunofluorescence assay 24 to 96-well plates.

**Protection of mice from replication of LCMV**

Mice were immunized once s.c. at the tail base with 0.3 μg gp33 plus 10 nmol CpG-ODN 1668, 33 μmol emulsified in IFA, CpG-ODN 1668 alone, or gp33 alone or were mock treated. Two weeks later, mice were infected i.v. with 200 PFU of LCMV, and viral titers in the spleens were determined after 4 days in a virus plaque assay, as described previously (28). LCMV was quantified with an immunofluorescence assay 24 to 96-well plates.

**Results**

**CpG-DNA renders CD8 T cell peptides immunogenic**

The use of CpG-DNA as an adjuvant appears to function by virtue of its direct DC-activating qualities (22, 23). We were interested to determine whether CpG-ODN-mediated activation of DC in vivo would render MHC class I-restricted peptides immunogenic for CD8 T cell precursors. C57BL/6 mice were challenged with SIINFEKL, either encapsulated in liposomes or as an aqueous solution, and were immunized on days 0 and 7 with a 2-fold dilution of virus mixed with CpG-ODN.

**Preparation of DC**

For preparation of DC, draining LN were removed and collected into ice-cold HBSS (Life Technologies, Karlsruhe, Germany). LN were digested for 1 h at 37°C using collagenase type Ia purchased from Sigma. Single-cell suspensions were prepared, and clumps were removed using a 100-μm pore size filter (Becton Dickinson, Heidelberg, Germany). Immediately after collagenase treatment, LN cells were washed in Ca2+-free HBSS, from this point onwards the cell suspension was always handled in buffers containing 2 mM EDTA. For positive selection of dendritic cells, single-cell suspensions were incubated with magnetic beads coated with the anti-CD11c Ab (Miltenyi, Bergisch Gladbach, Germany). Selection of DC was performed using MS separation columns and MiniMACS according to the protocol supplied by the manufacturer (Miltenyi). The selection ensured >90% pure CD11c+ cell population as judged by FACS analysis.

**Presentation assay**

Presentation of SIINFEKL was assayed by measurement of induced lacZ activity in SIINFEKL/Kb-specific T cell hybridoma (B3Z) transfected with a LacZ reporter under the transcriptional control of IL-2 gene promoter elements (26) after coinoculation with subpopulations of LN cells. Briefly, 12 and 24 h after SIINFEKL injection, the draining LN were harvested, and LN cells were separated into CD11c+ and CD11c− fractions as described above. Graded numbers of fractionated cells were incubated with 106 B3Z cells/well in 96-well plate at 37°C/5% CO2 overnight. The next day, individual cultures were washed with 100 μl PBS and lysed by addition of 100 μl Z buffer (100 mM 2-ME, 9 mM MgCl2, 0.125% Nonidet P-40, 0.15 mM chlorophenol red β-galactosidase (Calbiochem, San Diego, CA) in PBS). After 4 h incubation at 37°C, 50 μl stop buffer (300 mM glycine and 15 mM EDTA in water) were added to each well, and absorption of individual wells was read using a 96-well Enzyme plate reader (Molecular Devices, Sunnyvale, CA). The absorption wavelength was 570 nm, with 650 nm as the reference wavelength. Induction index was calculated by dividing induced activity by background.

**Flow cytometry**

Cells were washed in PBS containing 2% FCS (PBS/FCS) and first incubated for 10 min at 4°C with anti-CD3/anti-CD28/anti-CD40 and anti-CD8 Abs (Dynal, Hamburg, Germany) allowing negative selection of the CD4+ or CD8+ T cell subset, respectively. The efficiency of negative selection was >90% as judged by FACS analysis. The 51Cr release assay was performed as follows. EL-4 target cells (2 × 106) were labeled with 200 μCi Na235CrO4 (Amersham Pharmacia Biotech, Freiburg, Germany) for 1 h at 37°C and washed; one-half of 35Cr-labeled cells was incubated with peptide solution (0.1 μM SIINFEKL or 1 μM KAVYNFATM) for an additional 30 min. Peptide-untreated cells served as a specificity control. After extensive washing, 100 μl (106 cells) of target cells were added to the same volume of replicate serial dilutions of effector CTL. After 4 h of incubation at 37°C and 5% CO2, 100 μl of culture supernatant were removed from each well and γ-irradiation was measured. Specific lysis was calculated according to the formula: % specific lysis = [cpm (sample) − cpm (spontaneous release)]/[cpm (maximal release) − cpm (spontaneous release)] × 100. Spontaneous release ranged between 5 and 15%.

**Protection of mice from replication of LCMV**

Mice were immunized once s.c. at the tail base with 0.3 μg gp33 plus 10 nmol CpG-ODN 1668, 33 μmol emulsified in IFA, CpG-ODN 1668 alone, or gp33 alone or were mock treated. Two weeks later, mice were infected i.v. with 200 PFU of LCMV, and viral titers in the spleens were determined after 4 days in a virus plaque assay, as described previously (28). LCMV was quantified with an immunofluorescence assay 24 to 96-well plates. The LCMV-WE virus strain used in this study was originally obtained form Rolf Zinkernagel (University Hospital Zurich, Zurich, Switzerland) and was grown on L929 fibroblast cells with a low multiplicity of infection.

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ODN 1668. SIINFEKL-specific CTL could be generated in the class I peptide from LCMV (see below). To test protective immunity toward LCMV after immunization with an MHC class I peptide, mice were immunized with SIINFEKL (Ag) plus CpG-ODN 1668 (adjuvant). After 4 days, lymphocytes from the draining LN were harvested and cultured in vitro with 10 U/ml rIL-2 for an additional 4 days. Thereafter, lytic activity against EL-4 cells (C, V) or EL-4 cells pulsed with the SIINFEKL (●, ▼) was scored in a standard 51Cr release assay. Symbols represent CTL derived from individual mice. B. Before testing lytic activity in 51Cr release assay, SIINFEKL plus CpG-ODN-induced CTL were depleted of CD4+ or CD8+ cells. Targets were EL-4 cells (C, V) or EL-4 cells pulsed with the SIINFEKL (●, ▼). Symbols represent CTL derived from individual mice. A is representative of at least four experiments performed; B is representative of two experiments.

We also tested whether activation of CTL precursors is restricted to the draining LNs or becomes disseminated to a systemic level. To probe for the generation of primed T cells in the spleen, mice were challenged in the fore footpads with SIINFEKL (Ag) plus CpG-ODN 1668 (adjuvants). After 2 wk, their splenocytes were prepared and restimulated in vitro with the peptide SIINFEKL. As shown in Fig. 2B, strong SIINFEKL-specific lytic activity was generated, implying systemic dissemination of activated CTL. This conclusion is also supported by the induction of protective immunity toward LCMV after immunization with an MHC class I peptide from LCMV (see below).

Finally, we tested the effective dose range of the adjuvant CpG-ODN 1668. SIINFEKL-specific CTL could be generated in the draining LN in mice challenged locally with Ag plus 10–1 nmol CpG-ODN 1668 (data not shown).

Immature DC constitutively present SIINFEKL whereas CpG-DNA activates Ag-presenting DC
In situ, DC are believed to initiate primary CTL responses in a two-step process. After sampling and processing Ag as immature DC, they must subsequently mature into professional APC via activation (7). DC activation may be brought about by engagement of CD40L-positive Th cells (8–10) or via direct recognition of peptide-specific CD8+ T cells. These data suggested that in draining LN of wild-type mice, the peptide SIINFEKL activates CD8 CTL precursors in the presence of the immunostimulatory CpG-ODN 1668.

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pathogen-derived molecular pattern ligands (11). We analyzed whether immature DC within draining LN take up SIINFEKL and present the peptide in a H-2Kb-restricted fashion. CD11c+ DC from LN draining a local site of SIINFEKL challenge were positively selected and probed for H-2Kb-restricted peptide presentation. This was accomplished by using the SIINFEKL/Kb complex-specific T cell hybridoma B3Z, which is transfected with a LacZ reporter under the transcriptional control of IL-2 gene promoter elements (26). Within 12 h after s.c. challenge with soluble SIINFEKL, positively selected DC from the draining LN effectively presented SIINFEKL, in that the T cell hybridoma became activated (Fig. 3). The DC minus LN cell population, however, was strongly impoverished for the ability to activate the hybridoma. By 24 h after injection, SIINFEKL presentation by DC from draining LN began to decline. After injection of CpG-ODN 1668 alone, DC were unable to activate the hybridoma. On the other hand, challenge with SIINFEKL plus CpG-ODN 1668 generated slightly reduced SIINFEKL presentation when tested after 12–24 h. Whether this reflects activation of DC known to curtail the capacity of DC to take up extracellular ligands (29) needs to be analyzed. These data implied that SIINFEKL presentation was transient, DC derived, and CpG-ODN independent.

In a second step, the activation phenotype of the CD11c+ DC in LN was scored by FACS analysis. Costimulatory molecules (CD80, CD86, CD40) and MHC class II expression by DC from LN draining a SIINFEKL challenge remained immature and were indistinguishable from mock challenged DC (data not shown). Challenge with a mixture of SIINFEKL plus CpG-ODN, however, greatly augmented DC surface expression of costimulatory molecules and MHC class II (Fig. 4). DC activation is CpG-motif dependent, because ODN lacking a CpG-motif fail to activate DC (22, 23). Together, these data suggested that within the draining LN immature DC constitutively take up and present SIINFEKL. It is the CpG-DNA inoculation, however, that causes their activation and transit to professional APC.

CpG-DNA bypasses the requirement for T help in generating CTL responses to T cell peptides

Activation of Ag-presenting immature DC can be brought about by CD40L+ Th cells or by direct DC recognition of pathogen derived ligands. We analyzed whether CTL induction by peptides plus CpG-ODN was independent of T cell help. CTL induction by SIINFEKL plus CpG-ODN 1668 was tested in wild-type C57BL/6 mice and compared with that of haplotype matched CD40L−/− mice or Th cell-deficient MHC class II−/− mice. Fig. 5 shows that the magnitudes of SIINFEKL specific CTL responses in CD40L−/− and MHC class II−/− mice were similar to those in H-2Kb wild-type mice. These data suggest that CpG-DNA
allowed induction of Th cell-independent CTL responses toward MHC class I-restricted T cell peptides.

CpG-DNA allows T cell peptide to induce protective antiviral CTL responses

Murine LCMV infections are controlled by LCMV-specific CD8 CTL via perforin-dependent cytolysis of virus-infected cells (30). A major MHC class I-restricted T cell epitope for H-2b mice is the aa 33–41 peptide (gp33) derived from LCMV glycoprotein (31). This T cell epitope injected in IFA has been shown to induce protective immunity (32). We used this model system to determine whether CpG-ODN 1668 renders the MHC class I-restricted peptide gp33 immunogenic for CTL and whether the immunized mice are protected against LCMV infection. Draining LN of C57BL/6 mice challenged s.c. with an aqueous solution of gp33 plus CpG-ODN 1668 generated gp33-specific CTL (Fig. 6). The draining LN of mice immunized with gp33 alone, however, failed to do so.

These data demonstrate that CpG-DNA used as an adjuvant conveyed immunogenicity to the MHC class I-restricted T cell epitope gp33.

To analyze whether activation of gp33-reactive CTL by CpG-DNA is paralleled by protective immunity against LCMV infection, C57BL/6 mice were immunized once with gp33 plus CpG-ODN 1668. Control mice were immunized with gp33 or with CpG-ODN 1668 alone. For comparison, a group of mice was challenged with gp33 emulsified in IFA, an immunization protocol known to protect mice from LCMV infection (33). Two weeks after immunization mice were infected i.v. with 200 PFU LCMV, and 4 days later viral titers in spleens were determined. Pooled results for two independent experiments are shown. ○, viral loads in individual mice. ―, average virus titer in each group. ***, virus detection limit in the assay.

These data demonstrate that CpG-DNA used as an adjuvant conveyed immunogenicity to the MHC class I-restricted T cell epitope gp33.

Discussion

Adjuvants, such as CFA or IFA allow the induction of protective immunity against infections using defined T cell epitopes as vaccines (32, 34). The severe side effects caused primarily by the paraffin oil contained in these adjuvants precluded a detailed analysis of their mode of action (35) and their use in humans. There is accumulating evidence that CpG-DNA acts as a potent vaccine adjuvant for promoting Th-1-like immune responses (16–19, 36). In contrast to CFA and IFA, the side effects of CpG-DNA, such as toxic cytokine syndrome, appear to be transient (37). We therefore considered CpG-ODN useful as a model to study the mechanisms of adjuvant action. Here we demonstrate that CpG-DNA activates in situ immature Ag-presenting DC to transit to professional APC that are able to activate CTL precursors in the absence of T help.
In the case of LCMV peptide gp33, activation of CTL was paralleled by the induction of protective antiviral immunity. These data implicate CpG-DNA as a potential powerful adjuvant in vaccination protocols toward infectious and perhaps tumor-associated diseases.

MHC class I-restricted T cell epitopes (Ag) admixed with CFA or IFA have been shown to prime peptide-specific CTL responses (32, 34), implying presentation of T cell epitopes by APC. To address the question of whether upon s.c. challenge soluble T cell epitopes become presented by DC within draining LN, DC from such LN were scored ex vivo for H-2Kb-restricted peptide presentation. Within 12 h after peptide challenge, positively selected immature DC presented the OVA peptide SIINFEKL, activating the SIINFEKL-specific, H-2Kb-restricted T cell hybridoma, whereas the DC negative population did not (Fig. 3). Thus, it appears that uptake and presentation of peptide are constitutive functions of phenotypically immature DC (38). Others have pointed out a MHC class I presentation pathway for cytosolic delivery of exogenous Ags in DC (39–41). Injection of peptide alone failed to induce productive CTL responses. Whether T cell peptides presented by immature DC are ignored by Ag-reactive T cells or induce tolerance (34, 42) needs to be analyzed. In contrast, coadministration of CpG-DNA activated Ag-presenting immature DC to transit to professional APC as defined by up-regulation of costimulatory molecules (CD80, CD86, CD40) and MHC class II (Fig. 4). The evidence for DC maturation is also supported by priming of peptide-reactive precursor CTL (Fig. 1) and by induction of cytokine production.3 The effects of CpG-ODN on immature DC within LN were CpG-specific, because control ODN were inactive.

Recently, a pivotal role of CD40 ligation in DC activation has been identified (6, 7, 43). CD40 ligation by CD40L-positive Th cells or cross-linking by anti-CD40 mAb caused Ag-presenting immature DC to transit to professional APC. Once matured DC could activate CTL precursors in a Th cell-independent fashion (8–10). Here we describe that similar to CD40 ligation, CpG-DNA also activates in situ Ag-presenting immature DC to transit to professional APC. There are additional parallels between DC CD40 ligation and pathogen pattern recognition receptor engagement. Pathogen-derived ligands like CpG-DNA and LPS or Th cell-dependent CD40L-CD40 interaction initiate in APC signal transduction via activation of c-Jun NH2-terminal kinase and p38, but not via the extracellular signal-related kinase (44–46). We propose that CD40 or the CpG-DNA receptor, despite being different receptors, trigger similar intracellular signal pathways. These similarities therefore mimic each other in functional outcome, i.e., in the activation/maturation of immature DC. One would thus predict a number of potential uses for CpG-ODN as adjuvants or immunomodulating agents (47–50). As shown here, CpG-ODN renders T cell epitopes immunogenic by activating Ag-presenting immature DC. Because activation of Ag-presenting DC by CpG-DNA bypasses the need for Th cells, productive CTL responses can be initiated merely by MHC class I-restricted T cell epitopes. This attribute could be helpful in cases where the Th cell compartment is compromised. Additionally, CpG-ODN represents a single, easily produced, and chemically defined reagent.

To determine whether the potential benefits may be of practical significance, we vaccinated H-2b mice with a mixture of MHC class I-restricted LCMV-derived gp33 peptide plus CpG-ODN. LCMV infections are controlled by LCMV-specific CTL (30). This model system allowed us to analyze whether CpG-ODN not only aids peptide-specific CTL induction but also confers antiviral protection. Indeed, mice challenged once with gp33 peptide admixed with CpG-ODN 1668 generated primary CTL responses and were also protected from a subsequent LCMV infection. During the completion of these studies Oxenius et al. (51) arrived at a similar conclusion by priming and boosting H-2b mice with CpG-ODN admixed with gp33.

The nature of the Ag is believed to control the induction of CD8 or CD4 T cell immunity, in that endogenous Ags are presented by MHC class I molecules whereas exogenous and endocytosed Ags are presented by MHC class II molecules (1). DC, however, have the capacity to generate MHC class I-restricted T cell epitopes from soluble proteins via TAP-dependent pathways. High rate macrophagocytosis and cell type-specific routes may deliver exogenous material to the cytosol and may account, at least in part, for the cross-priming capacity of DC (38–41). Here we show that DC from LN draining a site of soluble SIINFEKL-peptide challenge retain their immature phenotype but effectively present SIINFEKL in a H-2Kb-restricted fashion. DC-depleted LN cells, however, did not present SIINFEKL. The exclusivity of cell type for the ability to present SIINFEKL contradicts a simple loading of empty MHC class I heavy chains. Whether DC indeed are specialized to take up and to sample, via macrophagocytosis, extracellular peptides to deliver them in the MHC class I presentation pathway needs to be clarified.

The data described here define the adjuvanticity of CpG-ODN by their ability to activate Ag-presenting immature DC to transit to professional APC. Operationally, similar to CD40 ligation by Th cells or by anti-CD40 mAb, activated Ag-presenting DC initiate CTL responses in the absence of T help. Our data endorse CpG-DNA as an inexpensive and promising adjuvant for vaccination protocols directed toward infectious and perhaps tumor-associated diseases.

Acknowledgments

We thank Drs. N. Shastri (University of California, Berkeley, CA) and B. L. Kellsall (National Institutes of Health, Bethesda, MD) for providing the B32 hybridoma, Dr. S. Bauer (Technical University of Munich, Munich, Germany) for valuable suggestions about work with peptides, and M. Mayer and B. Villmow for excellent technical assistance.

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