

CpG-DNA-Mediated Transient Lymphadenopathy Is Associated with a State of Th1 Predisposition to Antigen-Driven Responses¹

Grayson B. Lipford,² Tim Sparwasser,³ Stefan Zimmermann,⁴ Klaus Heeg,⁴ and Hermann Wagner

Infections can influence concurrent and subsequent Th1 vs Th2 immune responses to Ags. Through pattern recognition of foreign unmethylated CpG dinucleotides, the vertebrate innate immune system can sense infectious danger and typically replies with a Th1-polarized adaptive immune response. We examined whether CpG-DNA exposure would influence subsequent responses to infection and soluble Ags. CpG-DNA injection led to local lymphadenopathy characterized by maintenance of cellular composition with some biasing toward elevated dendritic cell composition. Sustained local production of IL-12 and IFN- γ from dendritic cells and T cells was shown. Prior injection by up to 2 wk with CpG-DNA protected BALB/c mice from Th2 driven lethal leishmaniasis. CpG-DNA injection by up to 5 wk before soluble Ag challenge resulted in the generation of Ag-specific CTL, Th1 recall responses to Ag, and Th1-polarized Ag-specific Abs. Thus, CpG-DNA instigated a local predisposition for intense CTL responses and Th1-polarized immune responses to subsequent infections or Ag challenge. The induction by the innate immune system of a locally contained hypersensitivity could represent a capacitating immune reaction yielding rapid conditioned responses to secondary infections. *The Journal of Immunology*, 2000, 165: 1228–1235.

Epidemiological studies suggest an inverse correlation between infections and the development of Th2-driven atopic disorders (1, 2). Some childhood infections cultivate a type I Th1 cell immunological environment with IL-12, IFN- γ , and TNF as predominant cytokines. Because these cytokines inhibit the induction of Th2 responses, the absence of such infections might release Th2 immune mechanisms and thus promote atopic disorders. One implication would be that infections driving Th1 responses predispose for reduced Th2 responses to subsequent Ag challenge. Supporting this view, a retrospective study of Japanese children demonstrated an inverse relationship between tuberculin delayed-type hypersensitivity and atopy (2). Indeed in murine models, infection with mycobacteria suppresses allergenic sensitization to Ag (3, 4).

It has been established that the deoxynucleotide fraction of bacillus of Calmette-Guérin (BCG)⁵ preparations is immunostimulatory (5, 6). The vertebrate innate immune system, through pattern recognition of foreign unmethylated CpG dinucleotides (CpG-

DNA), senses infectious danger (7, 8). CpG-DNA stimulates macrophages and dendritic cells (DCs) to synthesize cytokines (IL-12, IL-18, TNF- α , IFN- α , and IFN- β), chemokines (macrophage-inflammatory protein-1 α and -1 β , monocyte chemoattractant protein-1) and to up-modulate costimulatory receptors (CD 40, CD86), thus promoting Th1-biased T and B cell responses to Ag (9–15). These events could be reproduced with CpG containing single-stranded oligonucleotides, which serve as a powerful adjuvant for the induction of Ag-driven Th1 responses (16–19). CpG-DNA administration concurrent with *Leishmania major* infection in BALB/c mice results in protection from the normally lethal Th2-mediated disease (20). Similar to BCG infection, CpG-DNA when given concurrently or post-allergen sensitization eliminates the Th2-driven response leading to airway eosinophilia and IgE production on allergen challenge (21, 22). Thus, exposure to bacterial DNA recapitulates some immunological aspects of live bacterial infection including Th1-biased responses.

We have previously shown that an injection of CpG-DNA leads to an increase in splenic cell count, granulocyte-macrophage CFUs and early erythroid progenitors (23). In consideration of potential long-term changes in immunocyte composition and activation status due to infections, we examined the ability of CpG-DNA to alter the immune systems responsiveness to Ag and infection. Here we show that in vivo exposure to CpG-DNA predisposes for extended periods of time the immune system to induce CTL and to bias for Th1 responses.

Materials and Methods

Animals, materials, and reagents

Female C57BL/6 and BALB/c mice were purchased from Harlan-Winkelmann (Borchen, Germany). Animals were used at an age of 8 to 12 wk. LPS and DNA from *Escherichia coli* and OVA were purchased from Sigma (Munich, Germany). DNA was heat denatured (95°C, 10 min) and sonicated (2 min) before use. Quil A (Spikoside) was purchased from Isotec Production (Lulea, Sweden). CpG-DNA was the oligonucleotide 1668,

Institute for Medical Microbiology, Immunology and Hygiene, Klinikum Rechts der Isar, Technical University of Munich, Munich, Germany

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² Address correspondence and reprint requests to Dr. Grayson B. Lipford, Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Trogerstrasse 32, Munich, Germany, 81675. E-mail address: G.B.Lipford@lrz.tum.de

³ Current address: Skirball Institute of Biomolecular Medicine, New York University Medical Center, New York, NY.

⁴ Current address: Institute of Medical Microbiology and Hospital Hygiene, Philipps University of Marburg, Marburg, Germany.

⁵ Abbreviations used in this paper: BCG, bacillus of Calmette-Guérin; ODN, oligodeoxynucleotide; DC, dendritic cell; LN, lymph node; Lipo-OVA, liposome-encapsulated OVA.

containing a "CpG-motif" marked with bold letters (5'-TCC-ATG-**ACG-TTC**-CTG-ATG-CT) and its control non-CpG-DNA (oligodeoxynucleotide (ODN) 1720: 5'-TCC-ATG-**AGC**-TTC-CTG-ATG-CT) (24, 25). Both ODNs were phosphorothioate stabilized and synthesized by TibMol-Biol (Berlin, Germany).

Infection model

We have previously described the use of CpG-DNA to cure murine leishmaniasis (20). *Leishmania major* promastigotes (strain MHOM/IL/81/FE/BNI) were grown in Click's/RPMI supplemented with 10% FCS on Novy-Nicolle-MacNeal agar. Promastigotes (2×10^6) were injected into the right hind footpad of BALB/c mice. In addition, 5 nmol CpG-DNA in 40 μ l per hind footpad were injected 2 h before infection or at either 7 or 14 days before infection. Swelling in both hind footpads was measured weekly with a metric caliper, the uninfected footpad serving as an internal control for non-infection-related swelling. From both values, the percent increase of thickness was calculated (20).

Mouse treatment and harvesting cells

To determine the long term effects of CpG-DNA on the cellular composition of lymph nodes (LN), CpG-DNA was injected s.c. into each hind footpad of C57BL/6 mice in low endotoxin water (Sigma) at a dose of 5 nmol/footpad unless otherwise noted. LNs were aseptically removed and collected into HBSS (Life Technologies, Gaithersburg, MD). LNs were digested for 1 h at 37°C with 400 U/ml collagenase type Ia (Sigma). Single-cell suspensions were prepared, and clumps were removed by forcing the cells through a 100- μ m pore size filter (Falcon, Becton Dickinson, Mountain View, CA). LN cells were washed in PBS containing 2% FCS and counted.

For preparation of DCs, draining LN cells were collected as above with the exception that after collagenase treatment LN cells were washed in Ca^{2+} -free HBSS and handled in buffers containing 2 mM EDTA. For positive selection of DCs, LN cells were incubated with anti-CD11c Ab-coated magnetic beads (MiniMACs) and selected according to the manufacturer's protocol (Miltenyi, Bergisch Gladbach, Germany).

To produce LN cells depleted for a given cell population, T cells, B cells, or DCs were removed from LN cells with magnetic beads (MiniMACs) coupled with Abs to CD4 and CD8, CD19 or CD11c respectively, according to the manufacturer's protocol (Miltenyi). To positively selected CD4^+ or CD8^+ T cells, LN cells were depleted of B cells, as above, and positively selected, as above, for CD4 or CD8. CD4^+ and CD8^+ T cell purity was ~95% as determined by FACS.

Flow cytometry

LN cells were first incubated for 10 min at 4°C with anti-FC γ R/II/III mAb (PharMingen, San Diego, CA) to block unspecific Ab binding. mAbs included anti-CD11c, -B220, -CD3, and -GR.1 and their isotype controls (PharMingen).

Cytokine production in presensitized LN

To determine the long range changes in basal cytokine production within local draining LN post-CpG-DNA sensitization, CpG-DNA was injected in low endotoxin water (Sigma) at a dose of 5 nmol/footpad s.c. into each hind footpad of C57BL/6 mice unless otherwise noted. After various time intervals, LN cells were prepared as above and placed in culture without addition stimulus. The cells were cultured in Clicks/RPMI supplemented with 10% (v/v) heat-inactivated FCS, 50 μ M 2-ME, and antibiotics (penicillin G and streptomycin sulfate, both at 100 IU/ml). After overnight culture, the supernatants were harvested and assayed for IFN- γ , TNF, or total IL-12 by ELISA according to the manufacturer's protocol (PharMingen). Type I IFN was accessed with a bioassay according to the method of Diefenbach et al. (26). Additionally, mRNA was immediately prepared from isolated CD4 or CD8 T cells and used for RT-PCR. Both IFN- γ and IL-12R β 2 expression was accessed in a semiquantitative PCR using β -actin as the housekeeping gene. Primers used for murine IL-12R β 2 and β -actin were as previously reported (20). Primers used for IFN- γ were: sense 73–93, AACGCTACACACTGCATCTTG; antisense 473–453, ATGAGCTCATTTGAATGCTTGG, modified from the work of Montgomery and Dallman (27).

Functional analysis of presensitized LN

C57BL/6 mice were preinjected with 5 nmol/footpad CpG-DNA s.c. into each hind footpad unless otherwise noted. In additional experiments, LPS (100 μ g), Quil-A (30 μ g), or non-CpG-DNA were preinjected. This was followed by OVA injection at the indicated times. Soluble OVA or liposomes containing OVA (300 μ g/100- μ l injection dose) were prepared as

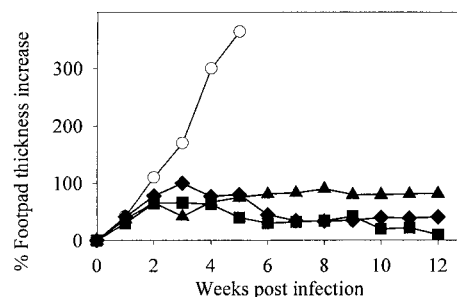


FIGURE 1. CpG-DNA predisposes for Th1-driven protection from lethal leishmaniasis. Groups of BALB/c mice were given CpG-DNA on days -14 (▲), -7 (◆), or 0 (■); an additional group was not given DNA (○). The mice were then challenged with 2×10^6 *L. major* promastigotes into the right hind footpad. The mean percent increase of footpad thickness is given ($n = 4$).

previously described (28). Four days post-OVA injection, draining popliteal LN cells were harvested as above. The LN cells were cultured for an additional 4 days in Click's/RPMI supplemented with 10% (v/v) heat-inactivated FCS, 10 U/ml recombinant human IL-2, 50 μ M 2-ME, and antibiotics (penicillin G and streptomycin sulfate, both at 100 IU/ml). OVA-specific CTL assays were performed as previously described (29). The targets were ^{51}Cr -labeled EL-4 cells, EL-4 cells pulsed with 100 nM SIINFEKL (the K ^{b} -restricted immunodominant peptide from OVA) or EG-7 cells (EG-7 cells are EL-4 cells transfected with the gene for OVA, gift from M. Bevan, University of Washington).

For Ab induction, C57BL/6 mice were injected with CpG-DNA in the hind footpads as above and then injected with 300 μ g/100 μ l OVA (50 μ l/footpad) at the indicated times. An OVA boost (300 μ g/100 μ l OVA, 50 μ l/footpad) was given 10 days after the initial OVA injection, and 1 wk later OVA-specific serum Ab titer was determined by Ab isotype-specific ELISA (16). Endpoint Ab titer was determined by coating 96F plates with 2 μ g OVA/well, incubating with serially diluted test sera and Ab detection with isotype-specific peroxidase-labeled Ab (PharMingen).

For Th recall assays, C57BL/6 mice were injected with CpG-DNA, as above, rested for various intervals, and injected with OVA. On day 7 post-OVA inoculation, LN cells were harvested, and 2.5×10^5 cells/ml were placed in 96-well culture plates with a restimulating dose of soluble OVA (100 μ g/ml). The supernatant was harvested, and cytokine ELISA was performed.

For adoptive transfer of in vivo presensitized DCs, positively selected CD11c cells were harvested from LN 7 days post-CpG-DNA injection as described above. The DC were pulsed with 50 μ g/ml OVA in vitro overnight, and 2×10^5 cells/50 μ l were injected into each hind footpad of naive C57BL/6 mice. LN cells were harvested from the injected mice and treated for Th recall as above. Cytokine levels were determined by commercially available ELISA kits (Genzyme (Cambridge, MA) or PharMingen). In additional experiments, the same transfer protocol was followed; however, popliteal LN cells were harvested 4 days after inoculation with OVA-pulsed DCs, and a CTL assay was performed as described above.

Results

CpG-DNA predisposes for Th1-driven protection from infection

In rodent models, it has been shown that BCG infection can inhibit the induction of Th2-driven asthma (3, 30). We asked whether preinjection of CpG-DNA, used to mimic BCG, would protect animals from Th2 responses. When BALB/c mice are infected locally with *L. major*, the resultant Th2-dominated response leads to nonclearance and death (20). BALB/c mice were injected at day -14, -7, or 0 with CpG-ODN before footpad infection with *L. major*. Fig. 1 shows that unprotected mice developed unchecked footpad swelling throughout the first 5 wk, whereas simultaneous CpG-ODN injection led to protection. Surprisingly, a day -14 or -7 preinjection also protected mice (Fig. 1). The protection was long lived, had the characteristics of a protective Th1 response to *L. major*, and the mice were protected from *L. major* reinfection

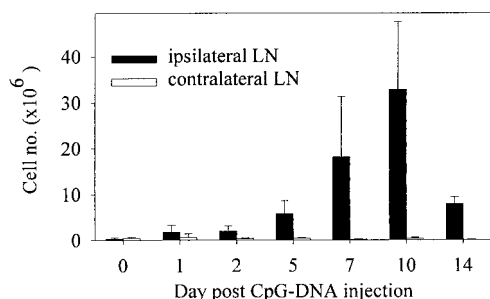


FIGURE 2. CpG-DNA induces local lymphadenopathy. Mice were injected with CpG-DNA in the hind right footpad. Both left and right popliteal LNs were extracted, and cellularity was assessed. □, left LN; ■, right LN ($n = 3$, mean + SD).

(data not shown). These results imply that injection of CpG-DNA alters the immune status, predisposing for a Th1-biased *Leishmania* clearance. We thus attempted to characterize what changes in immune status occurred post-CpG-DNA injection.

CpG-DNA induces local lymphadenopathy

We recently described that bacterial CpG-DNA induces extramedullary splenic hemopoiesis peaking at day 6 (23). In the course of these studies, not only splenomegaly but also lymphadenopathy restricted to the drainage field from the injection site was observed. To characterize the kinetics of lymphadenopathy, mice were injected in one hind footpad with CpG-DNA, and cellularity was recorded (Fig. 2). The ipsilateral popliteal LN experienced an increase in cellularity peaking ~10 days postinjection and contracting thereafter. In contrast, the contralateral LN did not change, appreciably reflecting the local nature of the response in the periphery and also serving as an internal control. Maximum cellularity in the local draining LN thus follows in time to that of the spleen, day 10 vs day 6, respectively (Fig. 2 and Ref. 23).

The day 10 LN cell number was 88-fold greater than that of uninjected mice (Table I and Fig. 2). Table I shows that the major cell types, B cells, T cells, DCs, and granulocytes, as defined by B220⁺, CD3⁺, CD11c⁺, or GR.1⁺ staining, respectively, all followed a similar kinetics of expansion and contraction although with notable exceptions. On day 1, B cells were underrepresented, however, CD11c⁺ cells were overrepresented severalfold above the total cellular increase. This outcome was to be expected if DC at the site of injection were activated by CpG-DNA compelling them to migrate toward the local lymphoid organs (14, 31). By day 2–5, B cells became overrepresented, correlating with CpG-DNA-induced B cell proliferation that peaked at day 2–3. B cell proliferation was tracked either histologically or in FACS, by Ki-67 or CD71 staining, respectively (data not shown). By day 7, the fold

increase of B220⁺, CD3⁺, and GR.1⁺ cells were similar to fold increase in total LN cellularity, but CD11c⁺ cells were still overrepresented. On day 10, at peak cellularity in the draining LN, the composition was normal with CD11c⁺ slightly overrepresented.

CpG-ODN predispose local draining LNs to respond to Ag

Because of the Ag presenting cell's (APC) critical role for productive T cell responses, we asked whether the numerical and compositional changes observed in the local draining LN would alter Ag-driven T cell activation. Previously, we described a vaccination technique that allows for the analysis of in vivo-generated primary cytolytic T cells; see *Materials and Methods* (28). Here we injected CpG-DNA into the hind footpads of mice, waited 7 days, and then inoculated the hind footpads with either liposome-encapsulated OVA (Lipo-OVA) or soluble OVA. Lipo-OVA induced mild CTL activity in non-DNA-sensitized mice (Fig. 3A). However, the response was greatly enhanced if the mice were "presensitized" with CpG-DNA. As expected, soluble OVA alone did not induce CTL, whereas CpG-DNA-presensitized mice generated a strong CTL response when inoculated with soluble OVA (Fig. 3A). Presensitization did not occur when a nonstimulatory control ODN, LPS, or the adjuvant Quil A was used (Fig. 3B). Thus, CpG-DNA presensitization predisposes for productive CTL responses to soluble Ags.

Injection of CpG-DNA triggers within the first 24 h a transient systemic release of cytokines (32). Elevated serum cytokines could be expected to mediate effects on distant lymphoid sites. Fig. 3C shows that the presensitization effect was restricted to the local drainage field. When Ag was injected contralateral to the DNA preinjection site, no CTL response could be measured. Additionally, i.p. CpG-DNA preinjection did not predispose the popliteal LNs to respond to the Ag (Fig. 3C). These data imply that CpG-DNA not only displays acute systemic effects but also presensitizes the local draining LN to respond to proteins.

Because the adjuvant effects of CpG-DNA are Th1 biasing (16–18), we tested whether CpG-DNA inoculation was Th1 predisposing. We presensitized mice at day –7 with CpG-DNA, injected Ag, and evaluated the Ab and Th responses. Fig. 3D shows that the serum Ab response to injected Ag in presensitized mice was both elevated and biased to IgG2a and IgG2b. This result was in agreement with the Th1-polarized response seen when CpG-DNA was injected simultaneously with Ag (16). Ab class switching to IgG2a is IFN- γ controlled; therefore, we tested the Ag-driven Th cell recall response in presensitized animals. C57BL/6 mice were presensitized at day –7 with CpG-DNA and then inoculated with OVA. Seven days later, the LNs were harvested, and an Ag-specific recall assay was performed. Fig. 3E shows that Ag induces a recall response resulting in IFN- γ production by cells harvested from CpG-DNA-presensitized mice. By ELISA, recall IL-4 production was

Table I. Fold increase for specific cell populations within the local draining LN post-CpG-DNA injection^a

Day	All Cells	B220 ⁺ Cells	CD3 ⁺ Cells	CD11c ⁺ Cells	GR.1 ⁺ Cells
0	1.00	1.00	1.00	1.00	1.00
1	4.68	2.24	5.59	23.87	5.94
2	5.22	7.86	3.71	21.87	4.65
5	15.30	20.39	12.76	56.63	15.27
7	48.76	39.16	54.02	134.72	65.21
10	88.70	71.39	97.57	331.47	83.10
14	21.22	18.55	20.81	134.93	30.65

^a Cells prepared from mice in Fig. 2 were stained for the surface markers listed and analyzed by FACS. Specific cell subtype numbers were calculated by multiplying the percent positively stained cells by the total number of cells extracted from the LN. With noninjected mice (day 0) as baseline, division of the cell number harvested from the various LN by the baseline number yielded a fold increase.

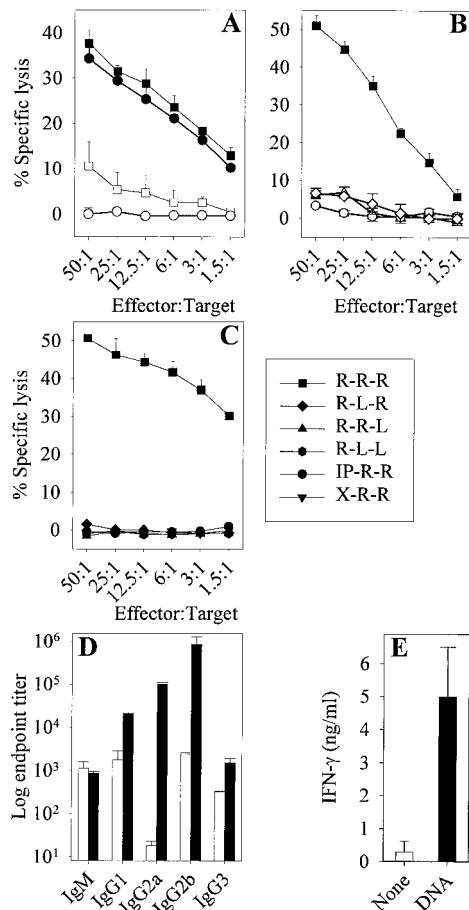


FIGURE 3. CpG-DNA predisposes local draining LNs to respond to Ag. **A**, Mice were mock injected (○, □) or injected with CpG-DNA (●, ■) into the hind footpads, rested 7 days, and inoculated with OVA in the same footpads. The mice were injected with either Lipo-OVA (□, ■) or soluble OVA (○, ●), and CTL assays were performed. The results are representative of several experiments. Intraassay variation, $n = 4$, is shown as $\pm$ SD. **B**, LN predisposition was restricted to CpG-DNA. CpG-DNA (■), LPS (○), Quil A (◇) or non-CpG-DNA (△) was injected 7 days before OVA injection and followed with a CTL assay. **C**, LN predisposition was restricted to the local drainage field. Mice were injected with CpG-DNA at various sites, OVA was injected at day 7 and followed with a CTL assay. The code for the legend is: site of CpG-DNA injection - site of OVA injection - site the LN cells harvested from for CTL, X = No CpG-DNA, R = right hind footpad, L = left hind footpad, or IP = intraperitoneal. **D**, For Ab induction, mice were injected in both hind footpads with CpG-DNA (■) or PBS (□), OVA was injected on day 7, a OVA boost was injected on day 17, and serum was harvested on day 24. The serum was evaluated for OVA-specific Ab end point titers. The results are representative of three experiments, and the result is shown as mean $\pm$ SD, $n = 2$ mice. **E**, For assessment of Th polarization, mice were injected in the hind footpads with CpG-DNA (■) or PBS (□), injected with OVA on day 7, the draining LN cells harvested on day 14 and restimulated in vitro with OVA. IFN- γ in the culture supernatants was measured by ELISA. Values are given as mean $\pm$ SD, $n = 3$ mice.

not measurable in either nonsensitized or presensitized animals (data not shown). Additionally, it was determined that both CD4⁺ and CD8⁺ T cells contributed to Ag-specific recall IFN- γ production (G.B.L., unpublished data). This result suggests that both Th1 and T cytotoxic (Tc) 1 responses were generated. Presensitization of animals

with CpG-DNA thus fosters a predisposition toward T cell-generated IFN- γ responses to Ag and induces Th1-polarized Ab responses.

CpG-DNA presensitization kinetics of LN Ag responses

Because of the intensity and altered character of the observed Ag-driven responses, we next analyzed the kinetics of presensitization. Fig. 4A demonstrates the effect of day 1–7 CpG-DNA presensitization on the induction of CTL activity. Preinjection of CpG-DNA led to a biphasic response kinetics to subsequent Ag challenge. The CTL response is diminished at both day 1 and 2 preinjection time followed by a recovery and overshoot with peak activity at day 4. In multiple experiments, the phase of diminished response was always at day 1; however, the peak of augmented response varied between day 4 and day 6. The kinetics of nonresponsiveness corresponds to the early infiltration of DC into the local draining LN (Table I). The recovery correlates with the strong cellular infiltration observed after day 2 (Table I). These results demonstrate that time was required for the predisposed LN environment to be established.

We next established the time limits of presensitization by increasing the time between CpG-DNA and Ag injection. Mice were either simultaneously injected with DNA and Ag or injected with DNA, rested for 7, 21, or 35 days and then given Ag. The presensitization by CpG-DNA that allows the induction of Ag-specific CTL lasted in excess of 35 days (Fig. 4B). For Ab induction, both the adjuvant effects and the Th1 biasing of CpG-DNA could be monitored for in excess of 35 days (Fig. 4C). Th1 biasing could also be measured by Th cell recall to Ag (Fig. 4D). When animals were CpG-DNA presensitized for up to 21 days before Ag injection, the resultant responding T cells from draining LN produced IFN- γ in response to in vitro OVA rechallenge. These data were unexpected and together imply that the Ag responsive environment once established prevails for extended periods of time.

CpG-DNA in vivo presensitized LN CD11c⁺ cells transfer CTL and Th1 responses to naive mice

Adoptively transferring Ag-pulsed DCs induces CTL responses in naive mice (33, 34). Because DCs occupy a critical role in initiating adaptive immune responses, we asked whether CTL induction and Th1 predisposition could be adoptively transferred via 7-day-presensitized LN DCs. LNs were CpG-DNA presensitized in vivo for 7 days, and then CD11c⁺ cells were isolated from the swollen LN. These cells were pulsed with soluble OVA overnight and then injected into the footpads of naive syngeneic mice. Fig. 5A shows that adoptively transferred in vitro OVA-pulsed CD11c⁺ cells harvested from presensitized mice induced primary CTL in the local LN. The DCs from nonpresensitized mice were not effective in inducing CTL under these conditions. Although the specific lysis values were low, they were reproducible, and it must be emphasized that the assay determined primary CTL response. Fig. 5B shows that the OVA response generated in naive mice inoculated with nonsensitized CD11c⁺ cells developed a Th2-polarized recall response, in that high amounts of IL-4 and IL-10 but low amounts of IFN- γ were produced. On the other hand, the presensitized CD11c⁺ cells initiated a Th1-biased response, i.e., high IFN- γ and low IL-4 and IL-10. Thus, Ag-pulsed DCs from 7-day CpG-DNA-presensitized LNs were able to induce Ag-driven CTL and Th1 responses in naive animals but nonpresensitized DCs bias to Th2 responses. These data infer that in vivo-presensitized DCs were able to convey the information for CTL induction and Th1 biasing to a naive LN environment.

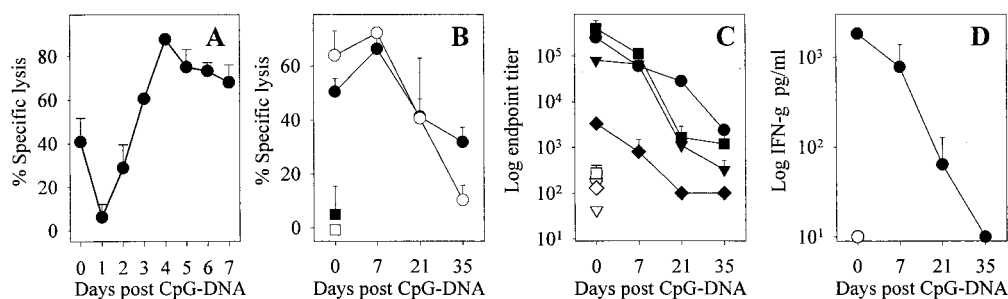


FIGURE 4. The kinetics of altered Ag response post-CpG-DNA presensitization. **A**, Mice were injected in the hind footpad with CpG-DNA, rested for the indicated time, and injected with OVA, and then a CTL assay was performed. ●, mean specific lysis + SD at the 50:1 ratio ($n = 2$ mice/time point). **B**, Mice were handled as in **A**. ○, ●, CpG-DNA-injected mice; □, ■, non-CpG-DNA-injected mice. ○, □, mice injected with soluble OVA; ●, ■, mice injected with Lipo-OVA. **C**, Mice were not injected (open symbols) or injected with CpG-DNA (closed symbols), injected with OVA on the indicated day, and boosted 10 days later, and serum was harvested 7 days later. Ag-specific Ab end point titers are shown for IgG1 (●), IgG2a (▼), IgG2b (■), and IgG3 (◆). Values are given as mean + SD, $n = 2$ mice/time point. **D**, Mice were not injected (○) or injected with CpG-DNA (●) and injected with OVA on the indicated day; the LN were harvested 7 days later and placed in culture with OVA. IFN- γ was measured by ELISA. Values are given as mean + SD, $n = 2$ mice/time point.

Basal IL-12 and IFN- γ are elevated in presensitized LNs

IL-12 and IFN- γ are involved in Th1 development. We reasoned that alterations in basal IL-12 or IFN- γ production could explain the presensitization. To determine basal cytokine production, we injected mice in the right hind footpad, harvested the ipsilateral and contralateral popliteal LN 7 days later, and cultured the dis-

sociated cells with no stimulus. Fig. 6, **A** and **B**, shows that the basal production of both IL-12 and IFN- γ were elevated in the draining LN and not the contralateral LN. TNF- α and type I IFNs were not detectable (not shown). Fig. 6C shows that basal IL-12 production in the ipsilateral LN remained elevated for at least 21 days. Detected by this method, IFN- γ was elevated only until day 7 and not detectable at day 14 (Fig. 6B and data not shown).

In an attempt to define the cells responsible for IL-12 and IFN- γ production from the 7-day LNs, we stained for intracellular cytokines followed by FACS analysis. Overall, this method proved too insensitive and thus inconclusive; however, small numbers of CD11c⁺ cells appeared positive for IL-12, and T cells were positive for IFN- γ if the TCR was cross-linked with anti-CD3 (data not shown). We therefore harvested 7-day presensitized LN cells,

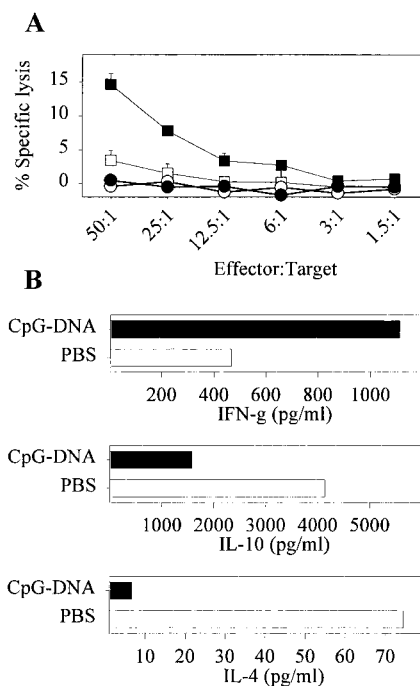


FIGURE 5. CpG-DNA in vivo-presensitized LN CD11c⁺ cells transfer CTL and Th1 responses to naive mice. C57BL/6 mice were injected in the footpads with CpG-DNA (closed symbols, closed bars) or PBS (open symbols, open bars). After 7 days, the draining LNs were harvested, CD11c⁺ cells were prepared and then pulsed with OVA. These cells were then injected into the hind footpads of naive C57BL/6 mice. **A**, After 4 days, the LN cells were harvested and cultured for an additional 4 days with IL-2, and a CTL assay was performed. The targets were EL-4 pulsed with the OVA-derived peptide, SIINFEKL (□, ■), or unpulsed EL-4 cells (○, ●). The symbols represent means + SE, $n = 2$ mice. **B**, In additional animals, LN cells were harvested after 7 days and cultured with OVA to assess Ag-specific recall by cytokine release. The results are representative of at least two independent experiments.

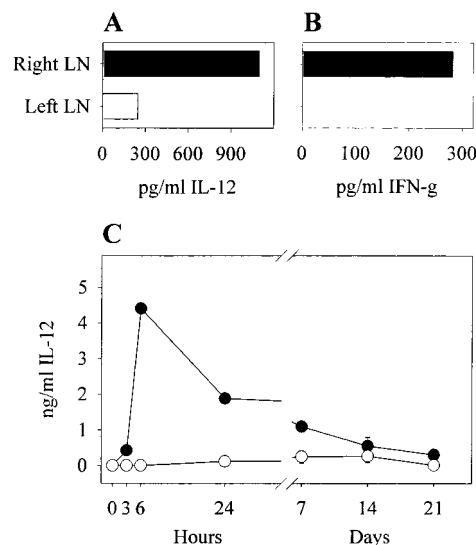


FIGURE 6. Presensitized local LN spontaneously produced cytokines. C57BL/6 mice were injected in the right hind footpads with CpG-DNA, rested 7 days, and the LN cells were harvested. The cells were prepared and cultured with no additional stimulus, IL-12 (**A**) and IFN- γ (**B**) levels were determined. **C**, To determine the persistence of elevated local cytokine production, mice were injected with CpG-DNA in the hind right footpad and rested for the indicated times; LN cells were harvested and placed in culture as above. Spontaneous IL-12 production was monitored from right popliteal LN cells (●) and left popliteal LN cells (○).

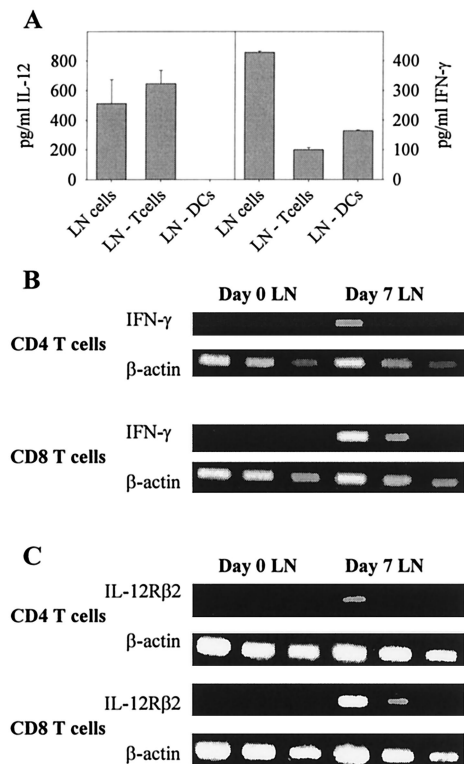


FIGURE 7. T cells and DCs from CpG-DNA sensitized LN are spontaneous producers of cytokines. **A**, C57BL/6 mice ($n = 4$) were injected in the hind footpads with CpG-DNA, and the LN cells were harvested at day 7 and pooled. The LN cells were depleted for CD11c⁺ or CD4⁺CD8⁺ cells, placed in culture with no stimulus, and assayed for cytokine release by ELISA. The values are presented as mean $\pm$ SD ($n = 3$ replicate wells), and the experiment is representative of two replicates. **B**, **C**, C57BL/6 mice ($n = 4$) were injected in the hind footpads with CpG-DNA, and the LN cells were harvested at day 7 and pooled. The LN cells were depleted of B cells and then positively selected for CD4⁺ or CD8⁺ T cells, and semi-quantitative RT-PCR was performed for IFN- γ (**B**) or IL-12R β 2 chain (**C**). The cDNAs were diluted in 4-fold steps; the experiment is representative of three independent experiments.

depleted for either CD4⁺/CD8⁺ or CD11c⁺ cells, cultured the remaining cells with no stimulus overnight, and assessed cytokine production as in Fig. 6 (Fig. 7A). IL-12 production was eliminated by the depletion of DCs. Depletion of either T cells or DCs reduced IFN- γ production (Fig. 7A). Thus, CD11c⁺ cells appear responsible for elevated basal IL-12, whereas the source of IFN- γ could not be restricted to a single cell type. When CD4⁺ or CD8⁺ T cells were purified and RT-PCR was performed, both cell types were positive for IFN- γ mRNA, whereas cells from nonpresensitized LN were negative (Fig. 7B). This result confirmed the potential involvement of T cells in elevated IFN- γ production but did not rule out DCs as a possible source.

Given that IL-12 and IFN- γ were elevated in CpG-DNA-presentation sensitized LNs, the altered environment could be expected to affect the composition of T cells. We purified CD4⁺ or CD8⁺ T cells and analyzed for IL-12R β 2 mRNA, a marker for Th1 cells. IL-12R β 2 mRNA was elevated in T cells from presensitized LN with a bias toward CD8⁺ T cells (Fig. 7C). Taken together, these data suggest that the elevated Th1-differentiating cytokine milieu found in the ipsilateral LN may at least in part explain the CpG-DNA presentation sensitization effects. The local environmental changes in baseline IL-12 and IFN- γ would be expected to influence T cell differentiation in response to subsequent infections or Ag inoculation.

Discussion

CpG-DNA appears to be a component of infection that acutely triggers innate non-self pattern recognition and thus the initiation of immune responses (7, 8). These responses are Th1 polarized and result in heightened hemopoietic activity that may be biased toward myeloid precursors (16–19, 23). Here we used CpG-DNA to probe whether pathogen-derived components sensitize and predispose mice for CTL and Th1 responses. We show that aseptic immunostimulation with CpG-DNA induced in mice local lymphadenopathy associated with long-lasting local immune hypersensitivity, which promoted CTL and Th1 responses to Ag and infection. This approach allowed us to temporally separate innate immune cell activation from Ag-driven adaptive immune responses. On first approximation, the results suggest that CpG-DNA triggers locally a lasting altered immune status which changes subsequent adaptive immune responses.

Recently, it was demonstrated that prior injection by up to 2 wk with CpG-DNA protected mice from lethal challenges with *Listeria monocytogenes* or *Francisella tularensis* (35–37). Serum IFN- γ levels were elevated for 24 h, and IFN- γ was claimed to be necessary for protection from *Listeria monocytogenes*. The serum levels of IL-12, on the other hand, were increased for at least 8 days after a single injection of CpG-DNA. In response to *F. tularensis*, B cells were critical for protection; however, both IL-12- and IFN- γ -deficient mice succumbed to infection (36). Whether the responses were Th1 or Th2 dominated was not evaluated. We show here that prior injection of CpG-DNA predisposes for a Th1-driven protection of BALB/c mice from Th2-driven lethal leishmaniasis (Fig. 1). The results suggest that extended IL-12 and IFN- γ production (Fig. 6) predisposes the immune response to Th1-polarized clearance of subsequent infection.

In the absence of adjuvants, challenge with soluble protein has been reported to induce T cell tolerance rather than productive T cell responses (38). CFA, rich in microbial components retained in an oil depot, is believed to simulate infectious non-self and to generate signals that activate APC (39, 40). During Ag presentation by APC to T cells, APC activation provides the secondary and tertiary signals necessary to overcome the inactivating signal of Ag only (41). Injection with soluble hen egg lysozyme failed to induce recall T cell proliferation or cytokine production; however, combination with IFA yielded a Th2 response, and CFA yielded a Th1 response (42). It was concluded that the addition of infectious non-self components to the adjuvant resulted in Th1-biased responses. Here we show that preinjection with CpG-DNA, a chemically defined APC-activating “infectious” signal, promotes a long lasting fertile ground for the induction of both Th1 and CTL responses to subsequent Ags injection (Figs. 3 and 4). In the absence of oils, both strong Th1 and CTL responses ensue in CpG-DNA-presentation sensitized animals.

A model has been proposed for the development of tolerance, Th2 or Th1 responses based on two thresholds in the magnitude of “infectious danger” signaling to innate immune cells (42). In essence, the model contends that APC activation attains three levels. When Ag is presented by nonactivated APC, tolerance develops, as expected during naturally developing self-tolerance. When the first threshold of activation is breached, costimulation occurs in the absence of IL-12, and Th2-like responses develop. When the second threshold is breached, in addition to costimulation, the innate immune system produces IL-12 and causes Th1 immunity. Here we show that LN cells from CpG-DNA presentation sensitized mice spontaneously produced both IL-12 and IFN- γ without further stimulation (Fig. 6). Depletion of CD11c⁺ cells from presentation sensitized LN cells eliminated IL-12 detection (Fig. 7A). Additionally, Ag-driven

Th1 induction in naive mice could be adoptively transferred by Ag-pulsed in vivo-presensitized CD11c⁺ cells (Fig. 5B). Taken together, these data demonstrate that DCs of the local draining LNs produce IL-12 for extended periods of time post-CpG-DNA injection and are sufficient to convey Th1 predisposition. If IL-12 is present at the time of T cell Ag stimulation, Th1 differentiation follows (43). It is thus not surprising that CpG-DNA-presensitized LNs respond to subsequent Ag or infectious challenge with polarization toward Th1.

IFN- γ positively modulates APC-presenting molecules and costimulatory molecules, enhancing Ag presentation and thus the likelihood of inducing productive T cell responses (44). We could not, however, detect the up-regulation of costimulatory molecules on DC after the first 3 days post-CpG-DNA injection (54). Alternatively, IFN- γ and possibly IL-12 positively influence DC maturation resulting from CD40-CD40L engagement (45–47). Both IL-12 and IFN- γ were continuously made in presensitized LNs (Fig. 6), and activated T cells were also present in the LN as judged from the detection of IFN- γ mRNA in the T cell population (Fig. 7B). The LN environment could thus provide the necessary queues for immature DC activation. Subsequent to Ag challenge, Ag-laden DCs would migrate into the local draining LN and be matured by activated bystander CD40L⁺ T cells and constitutively elevated IFN- γ and IL-12. This chain of events could explain the local hypersensitivity to soluble Ag.

CpG-DNA-sensitized LNs produced IL-12 and IFN- γ for extended periods (Fig. 6). IL-12 was produced by DCs, whereas both T cells and DCs appear partly responsible for IFN- γ production (Fig. 7). IL-12 can augment the production of IL-12 from DC and was recently shown also to drive DC IFN- γ production (48, 49). IL-12 and IL-18 can act together as a stimulus for IFN- γ production from differentiated Th1 cells through a TCR-independent mechanism (50, 51). Although we did not assay for IL-18, IFN- γ mRNA was expressed by resident T cells from CpG-DNA-presensitized LNs in the absence of an external Ag source (Fig. 7B). IFN- γ positively modulates the IL-12R β 2 chain to yield the high affinity IL-12 receptor capable of transducing the IL-12-driven signal (43, 52). We demonstrated that IL-12R β 2 chain mRNA was elevated in T cells from 7-day CpG-DNA-presensitized LNs (Fig. 7C). Whether the T cells expressing IL-12R β 2 mRNA were differentiated in situ or recruited to the LN as memory cells was not determined; however, it can be expected that these T cells should be responsive to environmental IL-12 and thus produce IFN- γ . The possibility arises that a positive, and perhaps autocrine, feedback loop between IL-12 and IFN- γ is self-perpetuating slowly diminishing with time. The maintenance of such an environment appears Ag independent and a product of the innate immune response, given that it was initiated by CpG-DNA without Ag-driven TCR stimulation.

If CpG-DNA represents a pattern recognition ligand that serves as an innate immune system stimulus signifying infectious danger, then the predisposition of local lymphoid organs to organize long term hypersensitive and Th1-polarized immune responses could mirror a biological consequence of infections. In analogy to a capacitor, elevated potential for activation would be stored locally. Once a primary infection compromises the first wall of defense, the epithelial barrier, challenge from secondary invaders would be more likely and perhaps at higher initial challenge dose. By lowering the activating threshold for the innate or adaptive immune system, responses driven by local hypersensitivity would be faster and more intense. In restricting hyperresponsiveness to the local environment, containment may be achieved, limiting the possibility of detrimental anti-self responses, whereas the cellular resources utilization would be minimized. Maintenance of a Th1-

predisposed local environment could be adaptive because most pathogen clearing responses are of a Th1 nature (53).

The idea of long term Th1-polarizing changes in the immune system has been postulated for humans; i.e., suppression of atopy was correlated with exposure and response to *Mycobacterium tuberculosis* (2). Because the propensity for atopic disorders is increasing in western societies, there is a need to determine the mechanisms by which Th1-polarizing infections change the signaling setpoints of the immune system, thus predisposing for subsequent Th1-biased responses. As shown here, the use of CpG-DNA may facilitate these investigations.

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