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*J Immunol* 1999; 162:2368-2374; ;
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Immunostimulatory CpG-Oligodeoxynucleotides Cause Extramedullary Murine Hemopoiesis1

Bacterial DNA and the synthetic CpG-oligodeoxynucleotides (ODNs) derived thereof have attracted attention because they activate cells of the adaptive immune system (lymphocytes) and the innate immune system (APCs) in a sequence-dependent manner. Here, we addressed whether CpG-ODNs affect hemopoiesis. Challenging mice with immunostimulatory CpG-ODN sequences led to transient splenomegaly, with a maximum increase of spleen weight at day 6. The induction of splenomegaly by CpG-ODNs was sequence-specific, dose-dependent, and associated with an increase in splenic cell count, in numbers of granulocyte-macrophage CFUs (GM-CFUs), and early erythroid progenitors (burst-forming units-erythroid). The transfer of spleen cells from CpG-ODN-pretreated animals into lethally irradiated syngeneic mice yielded an increase of spleen CFUs. Furthermore, the challenge of sublethally irradiated mice with CpG-ODNs caused radioprotective effects, in that recovery of GM-CFUs and cytotoxic T cell function was enhanced. The increase in GM-CFU and CTL function correlated with an enhanced resistance to Listeria infection in irradiated mice. We conclude from these data that CpG-ODNs trigger extramedullary hemopoiesis, and that this finding could be of therapeutic relevance in myelosuppression. The Journal of Immunology, 1999, 162: 2368–2374.

1 Abbreviations used in this paper: ODN, oligodeoxynucleotide; PTO, phosphorothioate; BM, bone marrow; m, murine; GM-CSF, granulocyte-macrophage CSF; h, human; GM-CFU, granulocyte-macrophage CFU; BFU-E, burst-forming unit-erythroid; CFU-S, spleen CFU.

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was purchased from Sigma (Munich, Germany). *Listeria monocytogenes* was obtained from strain 43251 from the American Type Culture Collection (Manassas, VA) and grown in a brain-heart infusion (Difco, Detroit, MI) in overnight cultures. The number of bacteria was determined by OD$_{600}$ and checked by plating 10-μl aliquots of a serial 10-fold dilution on Columbia blood agar plates and counting the CFUs after overnight incubation at 37°C.

**Treatment of mice**

CpG-ODNs were injected i.p. in low endotoxin water at 1–50 nmol/mouse; LPS was used at 10 μg/mouse. Negative control mice received injections with water alone. Sublethal irradiation of mice (4 Gy) was performed using a $^{60}$Co irradiator (Buchler, Braunschweig, Germany). For the induction of OVA-specific cytotoxic T cells, liposomes containing OVA were prepared as described previously (22). Inocula containing liposome-entrapped OVA with Quil-A as adjuvant were injected into the hind footpads of C57BL/6 mice; draining lymph nodes were harvested after 4 days. The lymph node cells were cultured for 4 days with 10 U/ml rIL-2, and CTL assays were performed as described previously (22). For Listeria infection, 5 × 10^5 *Listeria*/mouse were inoculated i.p. in a volume of 300 μl of brain-heart infusion into sublethally irradiated mice (4 Gy) at day 14 after irradiation; survival was recorded for the following 30 days. In experiments using sublethally irradiated mice, ODN-protected mice received 10 nmol of CpG-ODN (CG1) within 30 min after irradiation i.p.; control mice were mock-treated (injection of low endotoxin water). Each experiment performed had 3–10 mice per group per timepoint.

**Histopathology**

At various timepoints after ODN injection, mice were killed by CO$_2$ asphyxiation. Selected tissues, including spleen, liver, lymph nodes, and bone marrow (BM), were removed. For a determination of splenomegaly, organs were trimmed of fat and contiguous tissues and weighed. Organ/body weight ratios were calculated. Tissues processed for microscopic evaluation were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned (5-μm sections), mounted on slides, and stained with hematoxylin and eosin.

**Cytokines**

A purified preparation of murine (m) recombinant kit ligand (rmhisKL) was kindly provided by Dr. R. Mailhammer (GSF, Munich, Germany). It had been expressed in *E. coli* and purified by affinity chromatography as described previously (23). rmIL-3 was produced by X63Ag8-653 myeloma cells transfected with retroviral vectors carrying the mouse IL-3 gene (24). In short-term proliferation assays with cytokine-dependent indicator cell lines (25), a final concentration of 1% (v/v) X63Ag8-653 supernatant equaled the effect of 10 ng/ml purified mIL-3 obtained from Bachem Biochemica (Heidelberg, Germany). rmGM-CSF was a kind gift of Immunex (Seattle, WA). Human rIL-6 (rhIL-6) was obtained from Genzyme (Boston, MA).

**Quantification of GM-CFU**

Individual spleen cell samples from mice were analyzed for GM-CFUs by a soft agar colony assay as described previously (26). In brief, the desired number of spleen cells (final concentration was usually 3 × 10^5 to 1 × 10^6 per ml) was added to the agar medium mixture, and 1 ml was added in triplicate to 35-mm diameter culture plates (Greiner, Nürtinngen, Germany). Before cell plating, a saturating amount of a pretested mixture of myeloid colony-stimulating factor, including rmhisKL, rmIL-3, and rmGM-CSF (50 μl/plate, respectively) was added to the plates, corresponding to final concentrations of 500 ng/ml hisKL, 5 ng/ml IL-3, and 25 ng/ml GM-CSF. After gelling of the agar medium at 4°C, the cultures were incubated for 7 days at 37°C in a fully humidified atmosphere of 10% CO$_2$ in air. Cellular aggregates containing ≥50 cells were scored as colonies.

**Burst-forming unit-erythroid (BFU-E) assay**

A commercially available (CellSystems Biotechnologie Vertrieb, Remagen, Germany) culture medium composition (MethoCult HCC-3340) was used that contained 0.9% methylcellulose in α-modified Eagle’s medium, 30% FBS, 1% BSA, 10^−4 M 2-ME, 2 mM l-glutamine, and 3 U/ml recombinant human erythropoietin. A total of 0.3 ml of cell suspension (containing 13.2 × 10^3/ml spleen cells) was added to this medium (2.7 ml/tube). The culture medium was further complemented with 100 μl of rmhisKL (stock: 10 μg/ml), 100 μl of rmIL-3 (stock: 1 μg/ml), and 100 μl of rhIL-6 (stock: 100 ng/ml) and carefully mixed with a syringe fitted with a 1.4×40-m needle. This resulted in a final concentration of 300 ng/ml hisKL, 30 ng/ml rmIL-3, 3 ng/ml rhIL-6, and 4 × 10^−3/ml spleen cells, which were plated in triplicate aliquots of 1 ml per petri dish (Greiner). The growth of erythroid colonies (>50 hemoglobin-containing cells) was scored after an incubation period of 9 days at 37°C in a humidified atmosphere containing 10% CO$_2$ in air.

**Flow cytometry**

Cells (5 × 10^5–10^6) were washed in PBS containing 2% FCS and incubated for 10 min at 4°C with anti-FcγRII/III Ab from Pharmingen (Hanburg, Germany) to block unspecific binding of the following Ab reagents. mAbs used at 5–20 μg/ml included mAb against B220, CD3, Mac-1, and Gr-1. FITC- and phycoerythrin-labeled Abs were also purchased from Pharmingen. Isotype controls included purified rat IgG2a, rat IgG2b, and hamster IgG (all from Pharmingen). Between all incubation steps (30 min, 4°C), cells were washed with PBS/FCS. FACS analysis was performed on a Coulter Epics XL flow cytometer (Krefeld, Germany), acquiring 10,000 events. FACS data were analyzed using WinMDI 2.6/FACS-software (public domain software, Scripps Research Institute, LaJolla, CA).

**Results**

**CpG-ODNs cause transient splenomegaly**

Mice challenge i.p. with ODNs display a dramatic splenomegaly (Fig. 1). Kinetically, spleen weight increases to a peak at day 6 and subsequently normalizes. As detailed in Table I, an injection of CpG-ODN (CG1 or CG2) significantly induced splenomegaly, whereas in control non-CpG-ODN-injected animals, spleens were not significantly different for mock-injected animals. Thus, murine splenomegaly was induced in a CpG motif-dependent manner and peaked at day 6 after injection.

CpG-ODN has been shown to induce B cell proliferation, with a maximum observed between days 1 and 3 after challenge (Refs. 28 and 29 and our unpublished data). Therefore, we addressed the question of whether the observed splenomegaly was due to CpG-ODN-induced B cell mitogenicity. Cell surface
phenotyping of splenic cells by FACS analysis revealed that the absolute frequency of B220<sup>+</sup> cells (used as B cell marker) was only marginally increased (Fig. 2). However, the most dramatic effect observed was a transient but significant increase at day 6 in the B220-CD3 double-negative compartment. Histologically, an increased number of large immature blasts and erythroblasts was detected, with a maximum at day 6, suggesting increased hemopoietic activity (Fig. 3).

**Splenomegaly is associated with extramedullary hemopoiesis**

In contrast to humans, mice display a basal hemopoietic activity in the spleen (30). To analyze whether CpG-ODN-induced splenomegaly correlated with increased splenic hemopoietic activity, we measured the number of granulocyte-macrophage progenitor cells (GM-CFUs) in the spleens of CpG-ODN-treated mice. There was a 7.4-fold increase in splenic GM-CFU numbers at day 6, reflecting the kinetics of total spleen cell number (Fig. 4, A and B). We also analyzed the induction of GM-CFUs in BM from treated mice. There was a slight increase in the number of GM-CFUs in BM (day 4) that preceded the splenic increase at day 6, as if a mobilization of BM-derived progenitor cells to the spleen may have taken place (Fig. 4C). In addition, we enriched the B220/CD3 double-negative cell fraction from day 6 spleens of CpG- or non-CpG-treated mice by immunomagnetic separation and tested for GM-CFU formation. These cells were shown to be highly enriched for myeloid progenitor cells (Fig. 4D). Thus, the dramatic increase of the non-B, non-T cell fraction at day 6 after CpG-ODN injection was accompanied by an increased number of GM-CFUs within the spleen.

The induction of splenic hemopoiesis was CpG-ODN dose- and sequence-dependent (Fig. 5; Table I). Sequences lacking the CpG motif (nCG) failed to induce extramedullary hemopoiesis, suggesting that the presence of the CpG motif is necessary for the induction of hemopoietic activity.

### Table I. Increased spleen weight and number of GM-CFUs after injection of CpG-ODN

<table>
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<th>Group</th>
<th>Weight (mg/g bw)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GM-CFU/Spleen × 10&lt;sup&gt;3&lt;/sup&gt;</th>
<th>GM-CFU/10&lt;sup&gt;6&lt;/sup&gt; Cells&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>Control</td>
<td>3.92 ± 0.27</td>
<td>1.20 ± 0.43</td>
<td>7.75 ± 2.75</td>
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<tr>
<td>CG1</td>
<td>6.84 ± 1.42</td>
<td>8.58 ± 2.52</td>
<td>28.50 ± 7.75</td>
</tr>
<tr>
<td>GC</td>
<td>4.36 ± 0.36</td>
<td>2.07 ± 0.57</td>
<td>12.50 ± 3.00</td>
</tr>
<tr>
<td>CG2</td>
<td>6.91 ± 1.89</td>
<td>4.47 ± 0.87</td>
<td>13.50 ± 2.25</td>
</tr>
<tr>
<td>nCG</td>
<td>3.95 ± 0.31</td>
<td>1.13 ± 0.24</td>
<td>6.75 ± 1.50</td>
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<sup>a</sup> Increased spleen weight induced by CpG-ODNs. CpG-ODNs (CG1, CG2) induced significant splenomegaly in mice (mean values of three C57BL/6 mice per group ± SD, student’s t test; p ≤ 0.05), whereas non-CpG ODNs (nCG) did not. Inversion of the CG-dinucleotide (GC-ODN) almost completely abolishes the effect of CG1. A comparison between ODN-treated (10 nmol/mouse) and mock-treated mice (injection with low endotoxin water) is shown.

<sup>b</sup> Number of GM-CFUs per spleen (mean values of triplicate values of three C57/BL/6 mice per group ± SEM).

<sup>c</sup> Number of GM-CFUs per 1 million cells (mean values of triplicate values of three mice per group ± SEM). Data represent one of three typical experiments.

**FIGURE 3.** Histological changes in spleens of CpG-ODN-injected mice. A histomorphological analysis (hematoxylin and eosin staining) of spleens in CpG-ODN-treated BALB/c mice at day 6 after injection (B) was compared with normal spleens at day 0 (A). Spleen tissue at day 6 after 10 nmol of CpG-ODN (CG1) shows large immature blasts and erythroblasts that are indicative of increased extramedullary hemopoiesis (magnification: large panel, ×41; small insert, ×165).
and CG inversion (GC-ODN) almost completely abolished the hemopoietic effects of ODN CG1. A single-shot injection of CpG-ODN also compared well with the documented hemopoietic activity triggered by LPS (Fig. 5) (19–21). In addition to the granulocyte-macrophage progenitors, the number of pure erythroid progenitors after CpG-ODN injection was also increased as determined by the number of BFU-Es per spleen (mean values ± SEM, one of three similar experiments) induced by CpG-ODNs were measured at day 6 after injection.

Thus, the transient splenomegaly observed in ssDNA-injected mice was CpG motif-dependent and associated with extramedullary hemopoiesis.

Increased number of splenic progenitor cells is measurable by CFU-S assay

CFU-S cells are capable of lodging in the spleen and forming macroscopic nodules at 11 days after adoptive transfer into the BM-ablated host. As shown in Fig. 7, a significantly enhanced number of CFU-S was detected in spleen cells taken from CpG-ODN-pretreated mice. CFU-S exhibit many characteristics of primitive hemopoietic stem cells, such as extensive proliferative capacity, the ability for self-renewal, and the capability of generating spleen colonies containing cells of multiple hemopoietic lineages that can rescue animals from lethal irradiation (31). Thus, these data suggested the possibility of reconstituting lethally irradiated mice by an adoptive transfer of CFU-S contained in the spleens of CpG-ODN-treated mice.

CpG-ODNs mediate radioprotective effects in myelosuppression

In contrast to hemopoietic progenitor cells forming colonies in agar assays, CFU-S are considered to be rather radioresistant (30). Because CpG-ODNs induce extramedullary hemopoiesis via the mobilization of CFU-S to the spleen, we analyzed whether CpG-ODNs could mediate radioprotective effects in sublethally irradiated mice. CpG-ODN challenge of sublethally irradiated mice (4

![FIGURE 4. CpG-ODN-induced changes in splenic cell number, number of splenic GU-CFUs, and number of BM GM-CFUs. A, Kinetics of CpG-ODN (CG1)-induced changes in splenic cell count (mean values of three C57BL/6 mice per timepoint ± SD). B, Evaluation of hemopoietic progenitor cells in the spleens of CpG-ODN-treated mice. The graph displays the number of GM-CFUs per spleen per timepoint (mean values of triplicate spleen cell cultures of three mice ± SEM). C, Frequency of GM-CFUs in pooled BM cells from three mice per timepoint. D, Increased number of GM-CFUs in the B220/CD3 double-negative spleen cell fraction. Spleen cells from four nontreated C57BL/6 mice and three CpG-ODN (CG1)-injected mice (± SEM) were pooled at day 6 after i.p. injection. A portion of these cells was depleted for B220−, CD4−, and CD8− cells; both non-depleted and depleted (d) spleen cells were analyzed for GM-CFUs by soft agar colony assay (one of two experiments).](http://www.jimmunol.org/)

![FIGURE 5. Dose titration of CpG-ODN. Three BALB/c mice were injected with CpG-ODN (CG1) at different concentrations (1, 10, and 50 nmol/mouse, gray bars); LPS (10 μg/mouse, filled bars), solvent (low endotoxin water, open bars), and GC-ODN (dark gray bars) served as negative controls. The increased numbers of spleen cells and GM-CFUs per spleen (mean values ± SEM, one of three similar experiments) induced by CpG-ODNs were measured at day 6 after injection.](http://www.jimmunol.org/)

![FIGURE 6. Increased number of BFU-Es induced by CpG-ODN. The spleen cells of mice treated i.p. with 10 nmol of ODN CG1 (filled bars) or solvent control (low endotoxin water, open bars) were plated in a methylcellulose-based colony assay at day 6 after injection and scored for the growth of hemoglobin-containing erythroid colonies after an incubation period of 9 days in vitro (one of two experiments, mean values of five C57BL/6 mice ± SEM).](http://www.jimmunol.org/)
mice resulted in an enhanced resistance to *L. monocytogenes*. Basically, similar results were obtained in an infection model using CpG-ODNs to compensate for radiation-induced damage of the immune system. Two experimental systems were chosen: 1) the induction of CTL responses to proteinaceous Ags (10), and 2) resistance to the intracellular pathogen *L. monocytogenes* (32). Mice were treated with CpG-ODNs within 30 min after sublethal irradiation (4 Gy), allowed to recover for 18 days, and subsequently immunized s.c. with OVA-containing liposomes plus Quil-A as an adjuvant (22). After 4 days, cells of draining lymph nodes were harvested, cultured for an additional 4 days, and assayed for OVA-specific CTL activity. As detailed in Fig. 8B lymphocytes, from CpG-ODN-treated irradiated mice displayed an enhanced CTL response compared with nontreated irradiated mice. Basically, similar results were obtained in an infection model using *L. monocytogenes* (Fig. 8C). CpG-ODN challenge of irradiated mice resulted in an enhanced resistance to *L. monocytogenes* infection at day 14. Overall, the data given in Fig. 8 imply a correlation between CpG-ODN-induced extramedullary hemopoiesis and the ability to mount cytotoxic T cell responses or protective immune responses toward bacterial infections. We conclude that CpG-ODNs compensate for radiation-induced damage of the lymphohemopoietic system by accelerating regeneration from hemopoietic progenitor cells.

**Discussion**

In this study, we describe and characterize extramedullary hemopoiesis induced by CpG-ODNs. Mice challenged with CpG-ODNs develop a transient splenomegaly peaking at day 6 that is associated with increased splenic frequencies of B220/CD3 double-negative cells. Within this subset, hemopoietic progenitor cells were detected by GM-CFU and BFU in vitro assays. CpG-ODNs shorten the period of radiation-induced myelosuppression by improving hemopoietic regeneration via enhanced CFU-S export to the spleen. As a consequence, recovery of cytotoxic T cell responses and resistance to bacterial infection developed earlier in time after sublethal irradiation.

Bacterial DNA and CpG-ODNs polyclonally activate B cells and stimulate APCs such as dendritic cells and macrophages (4–8, 12). CpG-ODNs activate dendritic cells and macrophages in vitro to secrete large amounts of hemopoietically active cytokines, including IL-6, GM-CSF, IL-1, IL-12, and TNF-α (Refs. 5–7, 12, and 33–35, and our unpublished data). Mice challenged with CpG-ODNs also transiently exhibit high serum concentrations of these cytokines (6, 12). To date, it is unclear which of these cytokines, which are also known to act synergistically, triggers the extramedullary hemopoiesis described here. It is also conceivable that CpG-ODNs target BM stroma.
cells to release hemopoietically active cytokines. CpG-ODNs, which are operationally similar to LPS, may trigger extramedullary hemopoiesis via the induction of cytokines mobilizing BM progenitor cells to the spleen (19, 21).

Initially, we anticipated that the observed splenomegaly reflected CpG-ODN-induced B cell mitogenicity (4, 28, 29). However, it is only between days 1 and 4 after CpG-ODN challenge that proliferating B220+/CD3− cells account for the relative increase in splenic cellularity (Fig. 2; our unpublished data; and Ref. 29). Supporting a conclusion of non-B, non-T cell involvement in splenomegaly, spleen enlargement was also observed in SCID mice that lack B and T cells (data not shown). At day 6 after CpG-ODN challenge, B220+/CD3− splenic cells were prevalent (Fig. 2), and histology revealed abundant large, immature blast cells, which are indicative of extramedullary hemopoiesis (Fig. 3). In GM-CFU in vitro assays, the increased hemopoietic activity could be attributed to the B220+/CD3− population (Fig. 4D). In vitro colony assays (Figs. 4, 5, and 6; Table I) demonstrated a massive increase in splenic numbers of granulocyte-macrophage, and early erykroid progenitor cells. In peripheral blood, however, changes were discrete, in that leukocytosis and a slight reduction of numbers of erythrocytes and platelets were observed (data not shown).

It is known that bacterial stimuli (LPS or CFA containing heat-killed mycobacteria) can trigger increased splenic hemopoiesis (19, 21, 36), possibly via macrophage-derived hemopoietic growth factors that stimulate the generation and mobilization of the blood cells necessary to combat bacterial infections (reviewed in Ref. 30). Here, we show that CpG-ODNs, which are known to mimic the immunostimulatory effects of bacterial DNA (4), displayed the capacity to potentiate hemopoiesis. It should be noted that PTO-ODNs with and without CpG motifs can cause splenomegaly in rodents (28, 29, 37). This side effect of PTO-ODNs at very high doses appears to be caused predominantly by B cell proliferation. However, at the concentrations used here non-CG-PTO-ODNs were without effects (Table I; Fig. 5). Furthermore, bacterial genomic DNA causes splenomegaly, albeit less efficiently (our unpublished data).

CpG-ODN was shown to enhance hemopoietic regeneration from myelosuppression as caused by sublethal irradiation. For example, irradiated and CpG-ODN-treated mice exhibited increased numbers of splenic GM-CFUs, mounted Ag-specific CTL responses, and displayed enhanced resistance to L. monocytogenes infection (Fig. 8). The enhanced number of splenic GM-CFUs at 2 wk after injection of CpG-ODNs correlated with an enhanced immune system recovery in myelosuppressed mice. Hemopoietic depression and subsequent susceptibility to potentially lethal opportunistic infections are well-documented phenomena following chemotherapy, radiotherapy, or accidental radiation exposures. An inexpensive mitigation of myelosuppression would be of great clinical value. Our data suggest that CpG-ODNs can mitigate radiation-induced myelosuppression via an augmentation of hemopoiesis, yielding an accelerated reconstitution of the immune system.

Acknowledgments

We thank Dr. H. Baum from the Department of Clinical Chemistry (Technical University of Munich) for the analysis of murine peripheral blood and H. Broszeit and M. Mayer for excellent technical assistance. We also thank Dr. R. Endres for sharing unpublished data and Drs. Ch. Peschel and T. Decker for helpful scientific discussions and critical reading of the manuscript.

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