

## Effective Postexposure Treatment of Retrovirus-Induced Disease with Immunostimulatory DNA Containing CpG Motifs

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**Therapeutic strategies for the treatment of acute retroviral infections have relied mainly on antiviral drugs. In this study we used the Friend virus model system to demonstrate that enhancement of the immune system can also have dramatic therapeutic effects. Since resistance to Friend virus-induced leukemia in mice is associated with T helper cell type 1 (Th1) immune responses, we enhanced these responses in susceptible mice by treatment with synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN). Treatments begun at 4 days postinfection increased recovery from 6% in the control group to 74% in the CpG-treated group. CpG-mediated recovery was associated with a significant reduction of viral loads in the blood and spleens of treated mice compared to those of control animals. The treatment promoted Th1-type cytokine production by splenocytes of Friend virus-infected mice and augmented Friend virus-specific cytotoxic T-cell responses, but no influence on the virus-specific neutralizing antibody response was observed. Friend virus-specific CD8<sup>+</sup> T cells were critical for effective treatment with CpG-ODN, since *in vivo* depletion of these cells from treated mice prevented their recovery. Our results demonstrate that CpG-ODN therapy can significantly enhance virus-specific cellular immune responses and prevent retrovirus-induced disease. These findings may have implications for antiviral therapy in general.**

Due to the extremely high replication capacity of most viruses, the resolution of an infection and prevention of disease typically require rapid development of specific immune responses. The type of response generated is also very important, and the resolution of most viral infections is associated with type 1 helper T-cell (Th1) responses characterized by cytotoxic T-cell (CTL) activity and production of gamma interferon (IFN- $\gamma$ ) (27). The injection of synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN) has been shown to activate antigen-presenting cells in mice, which then promote IFN- $\gamma$  production by T cells and the induction of antigen-specific CTL (22, 35, 37). In addition, CpG-ODN enhance natural killer (NK) cell reactivity and positively influence antibody production by B cells (21, 41).

Thus, CpG-ODN have been used in a number of prophylactic studies in allergy models (40), for experimental cancer treatment (2), and as vaccine adjuvants (23, 25, 37). In particular, the ability of CpG-ODN to promote Th1 responses has already led to the design of phase I clinical trials with allergy patients (5). Furthermore, there is evidence from the infection of mice with *Leishmania major* that CpG-ODN may also have therapeutic value in infectious diseases by facilitating Th1-mediated immunity (43). These attributes suggested that CpG-ODN could be valuable for postexposure immune therapy of viral infections.

To test this, we analyzed the antiviral effect of CpG-ODN therapy following infection of mice with the Friend retrovirus.

Friend virus is a retroviral complex comprised of two components, a replication-competent helper virus called Friend murine leukemia virus, which is nonpathogenic in adult mice, and a replication-defective but pathogenic component called spleen focus-forming virus (20).

Infection of adult mice with Friend virus complex induces acute splenomegaly due to rapid polyclonal erythroblast proliferation, which is followed within several weeks by development of lethal erythroleukemia (17, 39). The pathogenic consequences of Friend virus infection are strongly influenced by the initial immune response of a mouse against the virus, which in turn is influenced by the major histocompatibility complex genotype of the infected animal. A comparison of the virus-specific immune responses of different mouse strains revealed that mice that are resistant to Friend virus-induced disease mount lymphocyte responses that appear earlier and are of higher magnitude than those of susceptible mice (13).

As in most other viral infections, the effective immune response against Friend virus is dominated by a Th1-type activation of the immune system, including the production of IFN- $\gamma$  and the activity of cytotoxic T cells (CTL) (8, 26). Here we show that a Th1-type response can be amplified by CpG-ODN during an initial retroviral infection to ultimately prevent the onset of lethal disease.

### MATERIALS AND METHODS

**Mice and virus.** Female (B10.A  $\times$  A.BY)F<sub>1</sub> mice (*H-2<sup>ab/b</sup>*) that were 3 to 6 months of age at the beginning of the experiment were used for all experiments. The B-tropic, polycythemia-inducing Friend virus complex used as the challenge virus in all experiments was from uncloned virus stocks obtained from 10% spleen cell homogenates as described previously (15). In all experiments, mice were injected intravenously with 0.5 ml of phosphate-buffered balanced salt

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solution containing 2% fetal bovine serum and 3,000 spleen focus-forming units of pathogenic Friend virus complex. After infection, mice were palpated for splenomegaly by observers blinded to the experimental status of the mice, as described previously (15).

**CpG-ODN treatment.** Phosphothioate-modified single-stranded oligodeoxynucleotides (ODN) were used in all experiments (MWG-Biotech AG, Ebersberg, Germany). The immunostimulatory sequence was 5'-TCCATGACGTTCTGATGCT-3' (referred to as CpG-ODN or CpG-1668), whereas the control ODN had inverted CG motifs (5'-TCCATGAGCTTCTGATGCT-3'). Mice were injected intraperitoneally with 15 nmol of CpG-ODN or control ODN in 0.5 ml of phosphate-buffered saline on days 4, 9, and 14 post-Friend virus infection.

**Analyses of viral loads in spleen and blood cells.** Infectious centers from spleens were detected by titrations of single-cell suspensions onto susceptible *Mus dunni* cells as described previously (6). For the quantification of Friend virus-infected blood cells, single-cell suspensions of nucleated, live cells were analyzed by flow cytometry. To detect Friend virus infection, cells were stained as described previously with tissue culture supernatant containing Friend murine leukemia virus glycosylated Gag-specific monoclonal antibody 34 (8).

**Cytokine-specific RNA protection assay and ELISA.** Spleen cells from Friend virus-infected mice were depleted of red blood cells, and  $10^7$  cells were stimulated with 2 ng of phorbol-12-myristate-13-acetate and 500 ng of ionomycin per ml for 5 h. Total RNA was isolated with Trizol (AppliChem, Darmstadt, Germany), and a commercial RNA protection assay (cytokine template set mCK-1; Pharmingen, Heidelberg, Germany) was performed according to the company's standard protocol. The quantity of protected RNA was determined with a PhosphorImager and ImageQuant software. For quantification, cytokine values were expressed as a percentage of the mean values of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each gel lane.

For the IFN- $\gamma$  enzyme-linked immunosorbent assay (ELISA),  $10^7$  splenocytes were stimulated with phorbol-12-myristate-13-acetate/ionomycin, and cell culture supernatant was collected 48 h later. The IFN- $\gamma$  concentration in the supernatant was determined with a commercial ELISA (OptEIA; Pharmingen) according to the instructions of the manufacturer.

**Friend virus-neutralizing antibody assay.** Heat-inactivated plasma samples from infected mice were incubated with virus stock in the presence of complement with or without  $\beta$ -mercaptoethanol to distinguish immunoglobulin G (IgG) from IgM as previously described (8). The samples were then plated on *M. dunni* cells to determine the dilution at which 75% of the virus was neutralized.

**Lymphocyte depletions.** CD8<sup>+</sup> T-cell depletions were performed essentially as described previously (15). Briefly, mice were inoculated intraperitoneally with 0.5 ml of supernatant fluid obtained from tissue culture for the CD8-specific monoclonal antibody 169.4. Mice were inoculated every other day four times, starting on the day of Friend virus infection. NK cells were depleted by two intraperitoneal injections of 50  $\mu$ g of NK1.1-specific antibody (Pharmingen) in 0.5 ml of phosphate-buffered saline. Injections were performed on days 0 and 4 post-Friend virus infection. The treatment depleted 95.2% of CD8<sup>+</sup> cells and 95.6% of NK1.1<sup>+</sup> cells in the blood of the respective mice (measured 1 week after the last antibody injection). At 2 weeks posttreatment of the different groups, the reduction in cells in the spleen was 73.4% for CD8<sup>+</sup> cells and 80.8% for NK1.1<sup>+</sup> cells. For flow cytometry analysis of depletions, cell populations were stained with anti-CD8a monoclonal antibody 5H19 (Caltag, Hamburg, Germany) or as the NK cell marker with the anti-DX5 monoclonal antibody (Pharmingen).

**IFN- $\gamma$ -specific Elispot assay.** The Elispot assay for the detection of IFN- $\gamma$ -producing splenocytes was performed essentially as described previously (26). Briefly, spleen cells from Friend virus-infected mice were depleted of red blood cells, serially diluted, and activated with Friend virus-infected spleen cells. No feeder cells were added to the cultures, and for the detection of spots, the AP conjugate kit (Bio-Rad, Munich, Germany) was used. The spots were counted with an Axioplan 2 microscope and the KS Elispot software (Carl Zeiss, Jena, Germany).

**Tetramer staining.** For detection of virus-specific CD8<sup>+</sup> T cells,  $5 \times 10^5$  nucleated spleen cells were dually stained with anti-CD8 (Ly-2) (Pharmingen) and phycoerythrin-labeled major histocompatibility complex class I H-2D<sup>b</sup> tetramer specific for the immunodominant GagL CTL epitope gPr80<sup>85-93</sup> (3) for 15 min at room temperature. The tetramers contained modified versions of the Gag epitope in which all three cysteine residues were replaced with aminobutyric acid (29). After washing, cells were analyzed by flow cytometry. Dead cells were excluded by propidium iodide staining. Since previous studies with Friend virus-infected susceptible mice (H-2<sup>b/b</sup>) revealed that the peak of the D<sup>b</sup>-GagL tetramer-positive CTL response occurs at 2 weeks postinfection (U. Dittmer and K. J. Hasenkrug, unpublished data), we compared quantities of CTL from CpG-treated mice and control animals at this time point.

## RESULTS

**CpG-ODN treatment prevented lethal disease in retrovirus-infected mice.** To investigate if CpG-ODN could be used as a therapeutic drug to augment Friend virus-specific Th1-type immunity sufficiently to prevent fatal Friend virus-induced erythroleukemia, we infected susceptible (B10.A  $\times$  A.BY)F<sub>1</sub> mice with a high dose of Friend virus; 94% of the mice injected with control ODN containing no CpG motif developed fatal Friend virus-induced erythroleukemia. In contrast, 74% of the mice treated with CpG-ODN 1668 on days 4, 9, and 14 post-Friend virus infection recovered from virus-induced splenomegaly and did not develop leukemia (Fig. 1a).

During the first weeks, animals of both groups developed splenomegaly, indicating acute infection. Onset of CpG-induced recovery was observed at 3 to 4 weeks post-Friend virus infection (Fig. 1). At the 4-week time point, 21 out of 30 CpG-treated mice had normal or only slightly enlarged spleens (size 1+ to 2+), whereas 25 out of 27 control mice presented with severe splenomegaly (size 3+ to 4+) (Fig. 1b). Thus, CpG-ODN treatment prevented leukemia in the majority of retrovirus-infected mice.

**CpG-ODN were effective in lowering viral loads in Friend virus-infected mice.** In order to ascertain whether the recovery from splenomegaly in CpG-treated mice correlated with reduced viral loads, we measured the level of Friend virus infection in the blood and spleens of all animals. At 4 weeks postinfection, the control mice harbored significantly more infectious centers in their spleens than mice treated with CpG-ODN (Fig. 2a). Significant differences between the groups were also seen in the level of infected blood cells, where control animals had 2.6 times higher levels than the CpG-treated mice (Fig. 2b). Reduction of spleen virus loads after CpG-ODN treatment was also observed at 8 weeks post-Friend virus infection (data not shown). However, at this time point, low levels of virus were detectable in all CpG-treated mice, indicating that the treatment reduced but did not completely prevent persistent infection.

**CpG-ODN promoted Th1-type immunity in Friend virus-infected mice.** To determine if the reduction of viral loads in animals treated with immunostimulatory CpG-ODN was due to an enhancement of antiviral immune reactions, we analyzed the capacity of CpG-ODN to induce a Th1-type milieu in Friend virus-infected mice. RNase protection assays were performed to compare cytokine mRNA levels in mitogen-stimulated splenocytes from treated and untreated mice at 4 weeks postinfection, the time point when recovery was observed (Fig. 1b). No differences between the two groups were observed in the levels of mRNA for interleukin-4 (IL-4), IL-5, IL-6, IL-10, IL-9, IL-13, or IL-15 (Fig. 3a and data not shown). In contrast, CpG-treated mice had IL-2 and IFN- $\gamma$  mRNA levels that were significantly higher than those from control ODN-treated animals (Fig. 3a). These data strongly suggested that the CpG-treated mice had enhanced Th1-type responses.

Since IFN- $\gamma$  plays a key role in recovery from Friend virus infection (26), we determined if increased IFN- $\gamma$  mRNA levels were associated with increased production of IFN- $\gamma$  protein by spleen cells. The mean IFN- $\gamma$  production by mitogen-stimulated splenocytes from CpG-treated mice was 7.6 times higher than that from control animals (Fig. 3b), indicating that CpG-

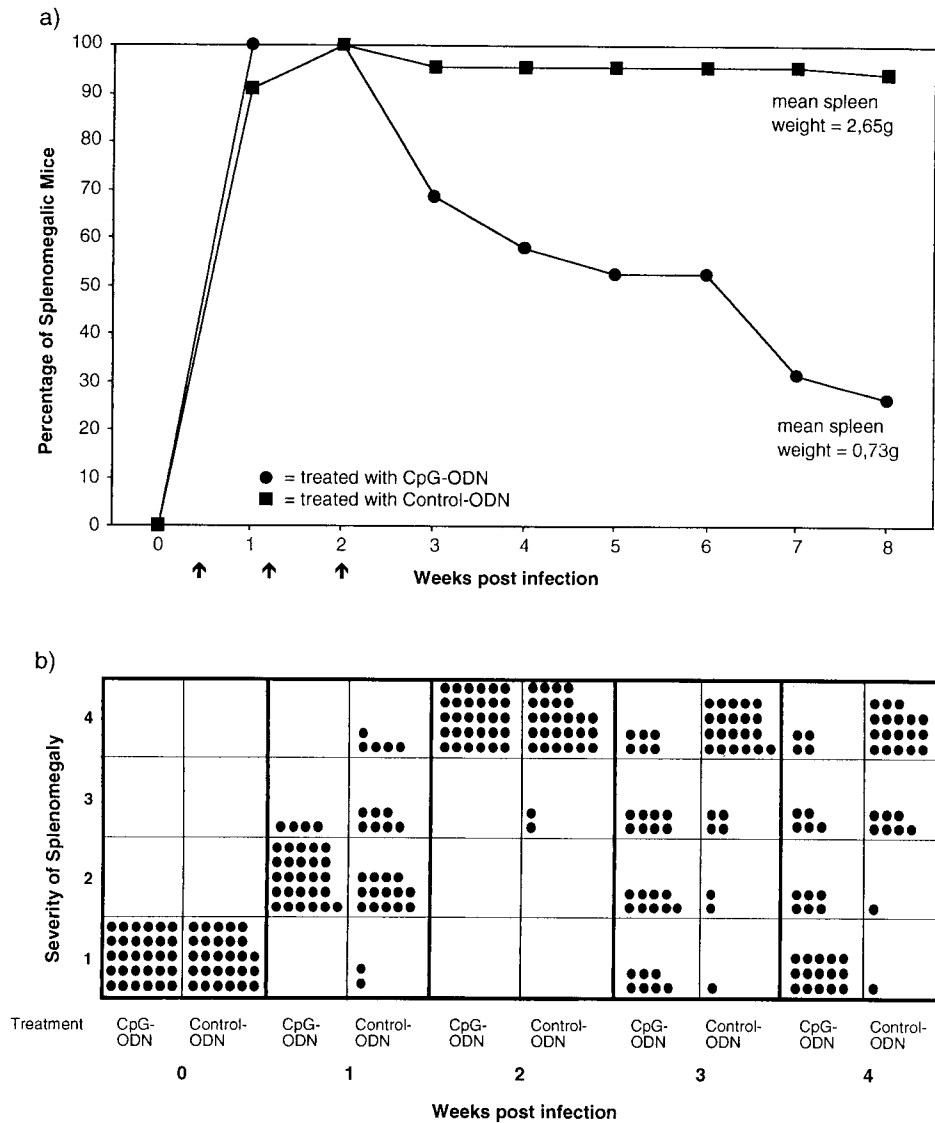


FIG. 1. Effect of CpG-ODN treatment on disease progression of Friend virus-infected mice. Adult age-matched female mice were inoculated intravenously with 3,000 spleen cell focus-forming units of Friend virus. Intraperitoneal injections of CpG-ODN or control ODN without the CpG motif were administered three times, on days 4, 9, and 14 postinfection (arrows). (a) Erythroleukemia induction and progression were followed for 8 weeks by spleen palpation as described previously (16). For CpG-treated mice,  $n = 19$ ; for control ODN-injected mice,  $n = 17$ . Results are from three independent experiments, which gave similar results (the number of CpG-treated mice in these experiments was 8, 5, and 6). The difference between the two groups was statistically significant by Fisher's exact test ( $P < 0.0001$ ). Mean spleen weights for each group at 8 weeks post-Friend virus infection are given in the figure. (b) Severity of splenomegaly in individual mice up to 4 weeks post-Friend virus infection. Animals were treated as indicated. Splenomegaly in individual mice was rated on a scale ranging from 1 (normal) to 4 (very severe) as described previously (16). Each increasing numerical value represents an approximate doubling of spleen size. Results are from five independent experiments.

ODN treatments directed the immune system of Friend virus-infected mice towards a protective Th1 phenotype.

**CpG-ODN augmented virus-specific CD8<sup>+</sup> T-cell responses but not virus-neutralizing antibody titers.** Since it has been demonstrated that IFN- $\gamma$ -producing Th1 cells influence virus-neutralizing antibody responses and CTL activity (4, 27, 34), we measured these responses to determine the effects of CpG-ODN treatment. No statistically significant differences in titers of Friend virus-neutralizing antibodies between CpG-treated and control animals were found (Fig. 4). This was true for the

Friend virus neutralization capacity of whole serum antibodies as well as for IgM-depleted, IgG subclass antibodies at 4 weeks postinfection.

To compare Friend virus-specific cellular immune responses in CpG-treated mice and control animals, we counted IFN- $\gamma$ -producing splenocytes after stimulation with Friend virus-infected cells in an Elispot assay. This assay has previously been shown to visualize Friend virus-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells (26), but NK cells might also be detected. CpG-treated mice had significantly more IFN- $\gamma$ -producing cells than ani-

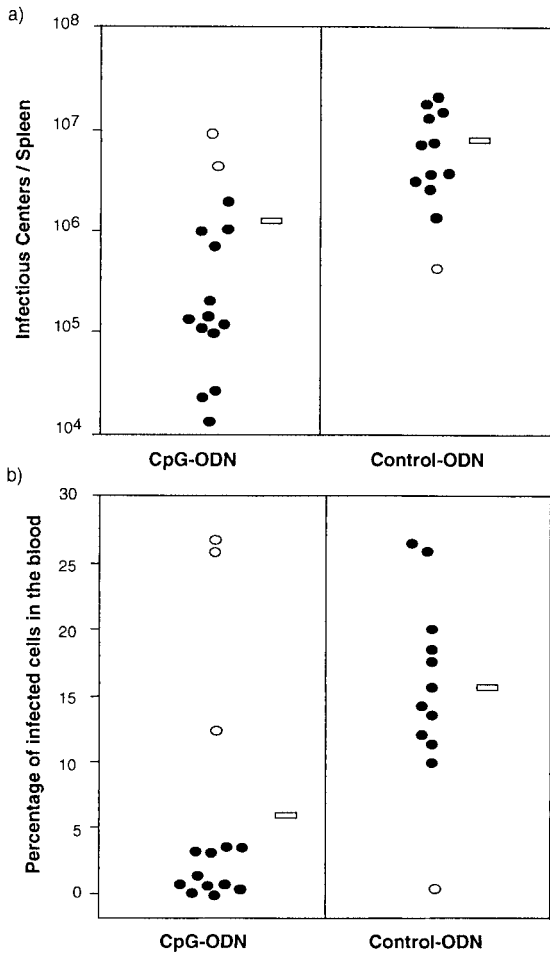


FIG. 2. Levels of Friend virus infection in the spleen and blood of ODN-treated mice. Cells from spleen or blood of mice taken at 4 weeks after infection were used to determine levels of Friend virus infection. In the CpG-treated group (left), the solid circles represent individual animals that responded to treatment and recovered from splenomegaly, whereas the open circles indicate therapy failures. In the control ODN group (right), the solid circles represent animals progressing to leukemia, whereas open circles indicate spontaneously recovered mice. (a) Single spleen cell suspensions were plated as infectious centers. The mean infectious center levels per spleen were, for the group of CpG-treated mice,  $12.2 \times 10^5$  (open bar on left) and, for the control group,  $77.1 \times 10^5$  (open bar on right). The differences between the groups of treated and untreated mice were statistically significant by Mann-Whitney test ( $P < 0.001$ ). (b) Percentages of blood cells expressing cell surface viral antigen. Live nucleated cells were stained for expression of Friend virus glycosylated Gag protein by flow cytometry. The mean percentages of blood infection were, for the group of CpG-treated mice, 5.9% (open bar on left) and, for the control group, 15.5% (open bar on right). The differences between the groups of treated and untreated mice were statistically significant by Mann-Whitney test ( $P < 0.01$ ).

mals receiving control ODN (Fig. 5a), indicating that CpG-ODN treatment could enhance virus-specific cellular immune responses.

In order to directly visualize Friend virus-specific CD8<sup>+</sup> T cells, we used the class I tetramer technique. The D<sup>b</sup>-GagL tetramers used were specific for the H-2D<sup>b</sup>-restricted, immunodominant gag CTL epitope gPr80<sup>gag</sup>85-93 (3). At 2 weeks

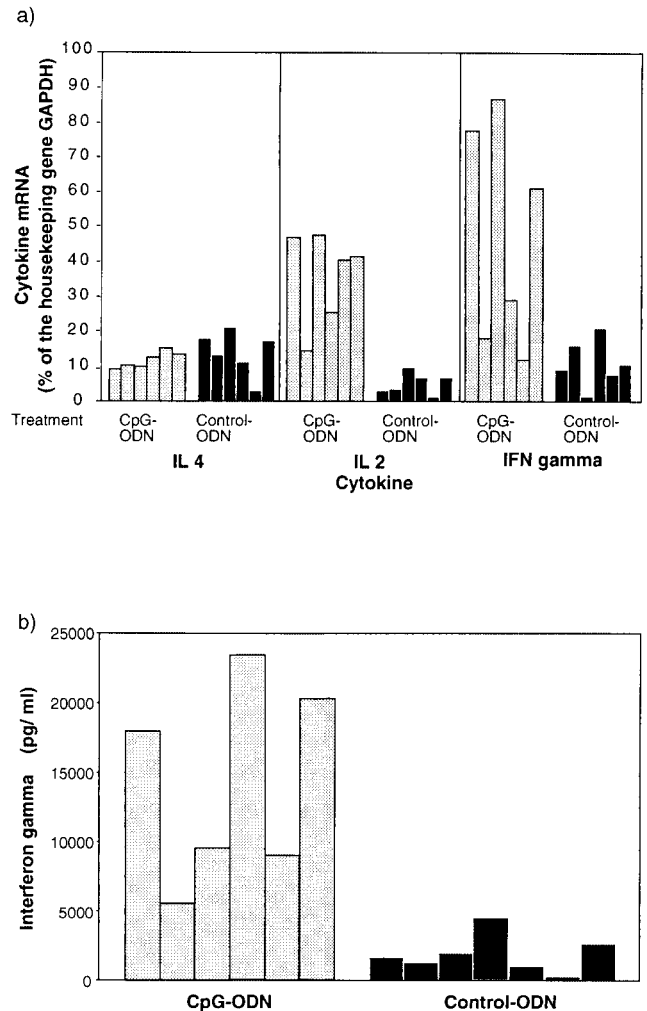


FIG. 3. Cytokine profile of splenocytes from Friend virus-infected mice after ODN treatment. (a) Levels of cytokine transcripts from splenocytes derived from Friend virus-infected CpG-treated mice (gray bars) and control ODN-inoculated mice (black bars) were compared by RNA protection assays. Cells were taken at 4 weeks postinfection and stimulated with phorbol-12-myristate13-acetate/ionomycin for 5 h. Band densities are expressed as percentages of specific cytokine band density of that of an internal housekeeping transcript band (GAPDH). Since CpG-ODN promote Th1 responses, only the typical Th1/Th2-type cytokines IL-2, IL-4, and IFN- $\gamma$  are shown. The differences in mRNA levels between the groups of treated and untreated mice were statistically significant by Mann-Whitney test for IL-2 ( $P < 0.005$ ) and IFN- $\gamma$  ( $P < 0.01$ ) but not for IL-4 ( $P > 0.05$ ). Additional cytokine mRNA levels which were analyzed but were not significantly enhanced or reduced in CpG-treated mice were those for IL-5, IL-6, IL-10, IL-9, IL-13, and IL-15. (b) IFN- $\gamma$  production from splenocytes of Friend virus-infected mice treated with CpG-ODN (gray bars) or control ODN (black bars). Cells were taken at 4 weeks postinfection and stimulated with phorbol-12-myristate13-acetate/ionomycin. After 48 h, supernatants were harvested and analyzed for IFN- $\gamma$  with a specific sandwich ELISA. The differences in IFN- $\gamma$  concentrations between the groups of treated and untreated mice were statistically significant by Mann-Whitney test ( $P < 0.005$ ). Each single bar represents an individual mouse.



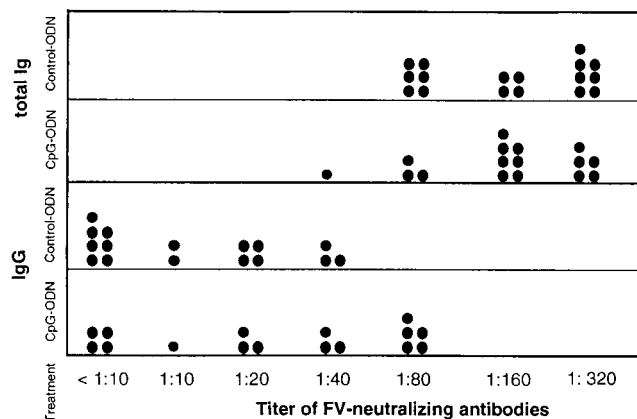


FIG. 4. Friend virus-neutralizing antibody titers in ODN-treated mice. At 4 weeks post-Friend virus (FV) infection, plasma samples from mice were analyzed for virus-neutralizing antibody. The neutralizing antibody titer was considered the highest dilution at which greater than 75% of the input virus was neutralized. Titers of total Friend virus-neutralizing antibodies (upper rows) and Friend virus-neutralizing IgG class antibodies (lower rows) are shown. Animals were treated as indicated. Each single dot represents an individual mouse. No statistically significant differences were observed between the groups by Student's *t* test ( $P > 0.05$  for total immunoglobulin and  $P = 0.05$  for IgG).

post-Friend virus infection, 2.5 to 2.9% of the CD8<sup>+</sup> T cells from control ODN-inoculated mice were positive for the D<sup>b</sup>-GagL tetramer. In contrast, three out of four CpG-treated mice had more than 5.5% CD8<sup>+</sup>/D<sup>b</sup>-GagL tetramer-positive T cells in the spleen (Fig. 5b). Thus, CpG-ODN therapy enhanced the Friend virus-specific CTL response in 75% of the mice, about the percentage of animals that recovered from Friend virus-induced splenomegaly after treatment.

To determine whether CD8<sup>+</sup> T cells were required for the curative effect of CpG-ODN, we depleted CD8<sup>+</sup> T cells in Friend virus-infected mice that were CpG treated. Animals that were depleted of CD8<sup>+</sup> T cells developed fatal leukemia despite the CpG-ODN inoculation (Fig. 6). This experiment showed that CD8<sup>+</sup> T cells were involved in CpG-induced recovery from retroviral infection.

In contrast, depletion of NK cells did not diminish the recovery of CpG-treated mice compared to a nondepleted control group (Fig. 6). This was somewhat unexpected, since NK cells have previously been associated with immunity to Friend virus (19). In addition, CpG treatment of mice increased the numbers of NK cells in their spleens and enhanced expression of the CD69 activation marker on NK cells in comparison to untreated control animals (data not shown). Thus, CpG-ODN were able to stimulate NK cells, but these cells did not appear to be required for the CpG-mediated effect. However, we cannot completely rule out that residual NK cells were still of some functional significance in the NK cell-depleted mice.

Taken together, the CD8<sup>+</sup> cell depletion experiment and quantification of the Friend virus-specific CTL suggested that the augmentation of CTL responses was critical for the antiviral effect of CpG-ODN.

## DISCUSSION

Here we describe the first use of CpG-ODN as postexposure treatment for a virus infection. The antiviral effect of CpG-ODN was obviously dependent on virus-specific CD8<sup>+</sup> T cells. This confirms data from our previous experiments, in which vaccine-induced CD8<sup>+</sup> T cells were able to prevent leukemia and reduce viral loads in the blood and spleen of Friend virus-challenged mice (7, 9). Thus, CD8 cells play an important role in Friend virus immunity, similar to what is known for other retroviral infections such as human immunodeficiency virus (HIV), simian immunodeficiency virus, and human T-cell leukemia virus type 1 (1, 30, 42). On the other hand, virus-neutralizing antibodies have also been shown to control Friend virus replication *in vivo* and induce recovery from splenomegaly (7, 9, 14). However, although CpG-ODN were known to stimulate antibody production by B cells (24, 43), we did not find enhanced titers of Friend virus-neutralizing antibodies in CpG-treated mice. Thus, there is strong evidence that the antiviral effect of CpG-ODN was mediated mainly by the cellular arm of the adaptive immune system. Specific T-cell responses can be augmented by Th1-type cytokines, such as IFN- $\gamma$  and IL-2, which were predominantly produced by splenocytes after CpG-ODN treatment of virus-infected mice. Since most viruses are controlled by Th1-type immune responses (27), CpG-ODN might have the potential to prevent pathogenic consequences in several viral infections.

Particularly for the treatment of HIV infections, researchers and clinicians are searching for ways to enhance HIV-specific immunity. There is strong evidence that an immunodeficiency virus-infected host who mounts a vigorous cellular immune response against the virus during acute infection can control its replication without medication and does not progress to disease (11, 28, 33). CpG-ODN treatment may be a simple and effective way to help promote the development of virus-specific immunity in individuals recently exposed to HIV. In addition, one major goal in AIDS research is to stimulate the HIV-specific immune responses during highly active antiretroviral therapy to make withdrawal of antiviral drugs with severe side effects possible (18). Since our results indicate that CpG-ODN are very interesting compounds for the stimulation of retrovirus-specific cellular immune responses, CpG-ODN may also be tested for their effect on the restoration of HIV-specific immunity in patients undergoing highly active antiretroviral therapy.

For such experiments, CpG-ODN that efficiently stimulate human or monkey lymphocytes would have to be used. The effect of CpG sequences and their flanking regions seems to be, at least to some extent, species specific. However, CpG-ODN that have an effect on monkey and human cells similar to that which CpG-ODN 1668 has on mouse cells have already been described (12). The immunostimulatory capacity of CpG-ODN is dependent on the frequency of CpG motifs in the ODN, the sequence of the region flanking the CpG motif, and the modification of the ODN backbone. CpG-ODN with sequences that efficiently stimulate NK cells were mainly used in cancer models (2). However, in most studies they could only provide anticancer activity when given prior to tumor challenge (2, 10).

To establish successful postexposure treatment against infectious agents, CpG-ODN that activate antigen-presenting

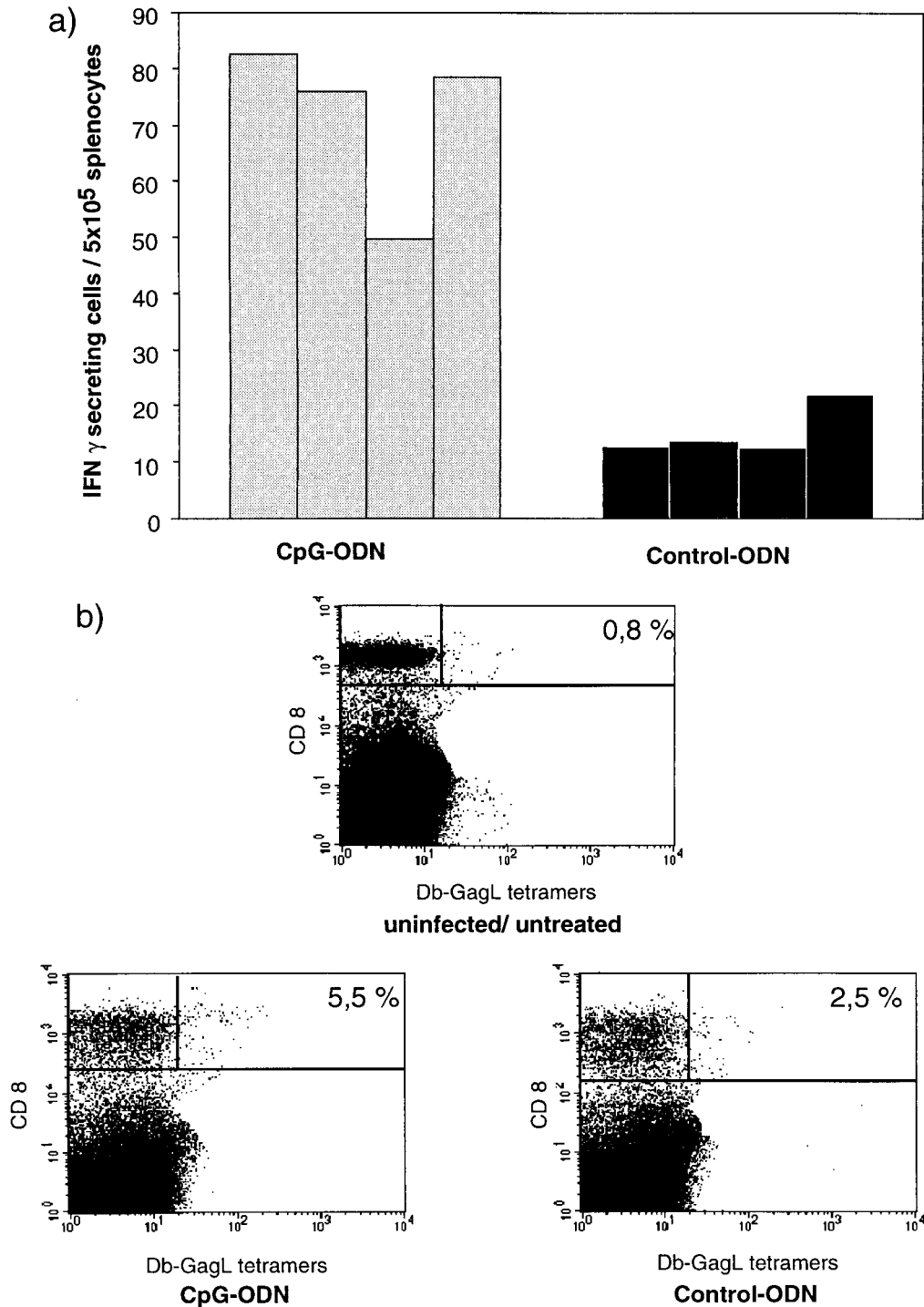


FIG. 5. Virus-specific T-cell responses in Friend virus-infected, ODN-treated mice. (a) Numbers of IFN- $\gamma$ -producing splenocytes at 4 weeks post-Friend virus infection in ODN-treated mice. Spleen cells from CpG-ODN (gray bars)- or control ODN (black bars)-inoculated mice were depleted of red blood cells, serially diluted, activated with stimulator cells, and analyzed for the number of IFN- $\gamma$ -producing cells with an Elispot assay. Animals were treated as indicated. Each single bar represents an individual mouse. The differences in numbers of IFN- $\gamma$ -producing cells between the groups of treated and untreated mice were statistically significant by Mann-Whitney test ( $P < 0.05$ ). (b) Representative flow cytometry profiles of Friend virus-specific CD8<sup>+</sup> T cells from Friend virus-infected ODN-treated mice. Live, nucleated cells were stained with anti-CD8 antibody and D<sup>b</sup>-GagL tetramers to detect Friend virus-specific CD8<sup>+</sup> T cells. The percentage shown in each dot plot is the percentage of D<sup>b</sup>-GagL tetramer-positive cells within the CD8<sup>+</sup> T-cell compartment (upper right quadrant). The complete results for the mice in the control ODN group were 2.7%, 2.5%, and 2.9%, and those for the CpG-ODN group were 5.6%, 5.5%, 7.1%, and 2.6% CD8<sup>+</sup>/D<sup>b</sup>-GagL tetramer-positive T cells. Similar to the control ODN-inoculated animals, untreated mice did not show more than 2.5% CD8<sup>+</sup>/D<sup>b</sup>-GagL tetramer-positive cells at 2 weeks post-Friend virus infection (data not shown). The background staining for uninfected mice was always between 0.5 and 1%. Dead cells were excluded by propidium iodide staining. Animals were treated as indicated.

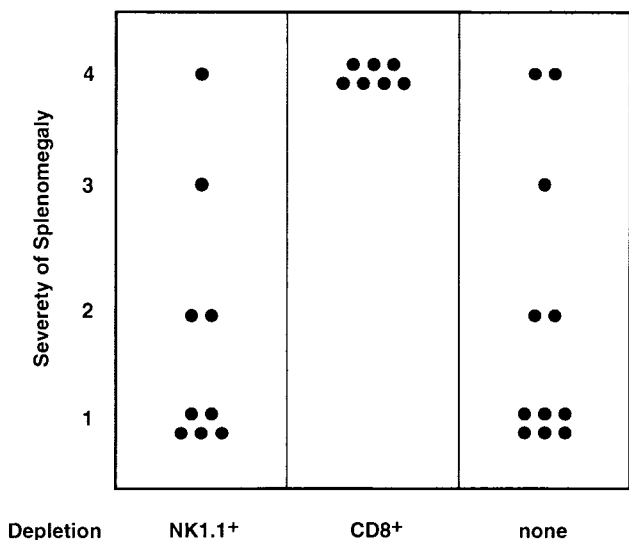


FIG. 6. Severity of splenomegaly in CpG-treated mice which were depleted of CD8<sup>+</sup> T cells or NK cells. Mice were infected with Friend virus and treated with immunostimulatory CpG-ODN. During acute infection, animals were depleted of CD8<sup>+</sup> T cells or NK cells as indicated and described in the text. At 5 weeks post-Friend virus infection, splenomegaly in individual mice was rated on a scale ranging from 1 (normal) to 4 (very severe) as described previously (16). Each increasing numerical value represents an approximate doubling of spleen size.

cells, resulting an enhanced T-cell responses, need to be identified. The CpG-ODN 1668 that we used in the current study has been reported to upregulate costimulatory molecules and major histocompatibility complex class I and II molecules on dendritic cells and to stimulate production of IL-12 by these cells (31, 32). Such molecules are critical for the recognition of viral antigens by T cells and were likely to be key players in augmenting Friend virus-specific CTL responses by CpG-ODN 1668. In a vaccine experiment, Vabulas et al. (36) have shown that CD8 cell epitope peptides from the lymphocytic choriomeningitis virus cannot be presented to CTL and do not directly induce CTL responses in mice. However, when CpG-ODN were used as adjuvants, peptide-loaded dendritic cells progressed to professional antigen-presenting cells and induced a protective antiviral CTL response.

Since many virus infections are controlled by CD8<sup>+</sup> CTL activity, CpG-ODN that stimulate antigen-presenting cells should be of general interest for the treatment of viral infections.

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