Immunomodulatory Effects of CpG Oligodeoxynucleotides on Established Th2 Responses

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CpG oligodeoxynucleotides (CpG ODNs) are known to induce type 1 T-helper-cell (Th1) responses. We have previously demonstrated that CpG ODNs administered during sensitization prevent Th2-mediated eosinophilic airway inflammation in vivo. We also reported that key Th1 cytokines, gamma interferon (IFN-γ) and interleukin 12 (IL-12), are not necessary for this protection. Recent in vivo data suggest that CpG ODNs might also reverse established pulmonary eosinophilia. In order to clarify how CpG ODNs can inhibit established Th2 responses, we evaluated the cytokine production from splenocytes from antigen- and alum-immunized mice. Restimulation with antigen induced IL-5, which was clearly inhibited by coculture with CpG ODNs in a concentration-dependent manner. CpG ODNs also induced IFN-γ, but in a concentration-independent manner. The inhibition of IL-5 production was not mediated through natural killer cells or via CD8+ T lymphocytes. Although IFN-γ plays an important role in inhibition of antigen-induced IL-5 production by CpG ODNs, IFN-γ was not the sole factor in IL-5 inhibition. CpG ODNs also induced IL-10, and this induction correlated well with IL-5 inhibition. Elimination of IL-10 reduced the anti-IL-5 effect of CpG ODNs, although incompletely. This may be because IFN-γ, induced by CpG ODNs, is also inhibited by IL-10, serving as a homeostatic mechanism for the Th1-Th2 balance. Overproduction of IFN-γ was downregulated by CpG ODN-induced IL-10 via modulation of IL-12 production. These data suggest that CpG ODNs may inhibit established Th2 immune responses through IFN-γ and IL-10 production, the latter serving to regulate excessive Th1 bias. These properties of CpG ODNs might be a useful feature in the development of immunotherapy adjuvants against allergic diseases such as asthma.

Recent epidemiological studies have demonstrated that the prevalence of allergic diseases, including asthma, is increasing worldwide (5). Therapy for these disorders is usually focused on relief from the symptoms. Once allergic diseases have been induced, chronic or seasonal medication is necessary. It has been recognized that CD4-positive T lymphocytes (CD4+ T cells) play a central role in the pathogenesis of atopic conditions (21). CD4+ T cells can be divided into the type 1 T-helper-cell (Th1) and Th2 types on the basis of the cytokines that they produce; these lymphocyte groups have counterregulatory effects on each other (26). Present understanding of allergic diseases suggests that their pathogenesis is due to strong Th2 immune deviation against otherwise benign specific antigens (allergens). At present, the most widely used antiallergy treatments are corticosteroids (4). Although steroids are often successful in minimizing the manifestations of inflammation, their use is not disease modifying or curative, and chronic therapy is needed to prevent pathological progression. Immunotherapy is a relatively uncommon alternative treatment for allergic diseases. Although its efficacy for asthma has been questioned, controlled studies support use of immunotherapy for many cases of allergic asthma (1,12). It is the only method of therapy that is thought to act, at least in part, through alteration of Th2 to Th1 immune responses against specific allergens.

DNA motifs containing a central unmethylated CpG dinucleotide in specific base sequences are known to trigger Th1 responses; these responses can be reprimed using oligodeoxynucleotides (ODNs) containing one or more CpG motifs (CpG ODNs) (19). It has previously been reported (8,24,39,41) that CpG ODNs can prevent antigen-induced asthmatic responses in mice. More recently, we have shown that in vitro antigen-recall challenges to splenocytes from mice sensitized to ovalbumin (OVA) along with CpG ODNs lead to the release of gamma interferon (IFN-γ) but not interleukin 5 (IL-5), and the converse response (IL-5 but not IFN-γ) is seen in splenocytes from mice sensitized in the absence of CpG ODNs (22). Those results indicated that CpG ODNs shifted the initial immune response against an antigen from Th0 to Th1. Interestingly, CpG ODNs also appear to be effective when administered as an immunotherapy adjuvant after sensitization (22,34,36). These data suggest that CpG ODNs are able to inhibit established Th2 immune responses against antigen, converting these to a Th1 immune response. It is known that the Th1 cytokine IFN-γ inhibits the synthesis of IL-4 and IL-5 by Th2 cells (43), suggesting the possibility that CpG ODNs inhibit established Th2 immune responses through IFN-γ. However, we recently reported that CpG ODNs can prevent Th2-mediated allergen-induced airway eosinophilia and airway hyperreactivity in the absence of the Th1 cytokines IFN-γ and IL-12 (23). On the basis of these data, we hypothesized that CpG ODNs can inhibit Th2 immune responses independently of the induction of Th1 cytokines. In order to test this hypothesis, we investigated the in vitro responses of splenocytes from sensi-
tized mice to antigen in the presence or absence of CpG ODNs.

**MATERIALS AND METHODS**

**Animals.** Six-week-old female C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice with a C57BL/6 genetic background and disrupted genes for IFN-γ and IL-12 (IFN-γ/IL-12-double-knockout [KO] mice) were bred in our animal facility (23). Mice with a C57BL/6 genetic background and disrupted genes for IL-10 (IL-10-knockout [KO] mice) were kindly provided Joel Weinstock (University of Iowa College of Medicine, Ames). All animal care and housing requirements of the National Institutes of Health Committee on the Care and Use of Laboratory Animals were followed.

**Reagents.** The CpG ODNs (ODN 1826; TCCATGAGCTTGTGAGGTT [where the boldface indicates the CG dinucleotide]), supplied by Coley Pharmaceutical Group (Wellesley, Mass.), were produced under good manufacturing practice conditions and had undetectable levels of lipopolysaccharide by the Limulus amebocyte lysate assay.

**MAbs and antisera.** To deplete natural killer (NK) cells and CD8-positive T lymphocytes (CD8+ T cells), an anti-mouse NK cell monoclonal antibody (MAb) (MAb DX5) and an anti-mouse CD8α MAb (MAB Ly2-2) were used (Miltenyi Biotec, Auburn, Calif.). To block the effects of IFN-γ, IL-12, transforming growth factor β (TGF-β), type I IFN (IFN-α/β), and IL-10, we used neutralizing MAbs and antisera. An anti-mouse IFN-γ MAb (MAB R4-6A2) and anti-mouse IL-12 MAbs (MAbs C15.6 and C17.8) were generously provided by Z. K. Ballas (University of Iowa College of Medicine). An anti-TGF-β MAb (MAB 1D11) and an anti-mouse IFN-α/β antiserum were purchased from R&D Systems (Minneapolis, Minn.) and Biosource International (Camarillo, Calif.), respectively. Rat immunoglobulin G1 (IgG1), rat IgG2A, rat IgG2b, and mouse IgG1 (all from BD Pharmingen) and sheep serum (Sigma, St. Louis, Mo.) were used as isotype control antibodies and antisera.

**Establishment of Th2 immune response in mice.** The mice were given intraperitoneal injections containing 10 μg of OVA (Sigma) mixed with 1 mg of aluminum hydroxide (alum) at 37°C in a volume of 0.2 ml of saline on days 0, 7, and 14.

**Culture of splenocytes.** The mice were killed with an intraperitoneal injection of 150 mg of pentobarbital sodium (Abbott Laboratories, North Chicago, Ill.) per kg of body weight on day 21. Single-cell suspensions of spleen cells were plated in 24-well tissue culture plates at a final concentration of 5 × 10⁶ cells/ml in RPMI 1640 supplemented with 2 mM glutamine, 25 mM HEPES, 10% heat-inactivated fetal calf serum, 50 mM 2-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. In some experiments, specific cells were depleted with antibody-coated beads through a depletion column with a magnetic particle concentrator (MACS system; Miltenyi Biotec) according to the instructions of the manufacturer. By this procedure, more than 99.9% of the labeled cells can be removed. The cells were incubated at 37°C in an atmosphere of 5% CO₂ in air. The cells were stimulated with OVA at a final concentration of 100 μg/ml. ODNs (either CpG or control ODNs) at final concentrations of 0.01, 0.1, and 1 μg/ml were added to the suspensions at the time of stimulation by OVA. The supernatants were harvested 72 h after culture and stored at −70°C until the measurement of cytokine levels. Percent inhibition of antigen-induced IL-5 production by CpG ODNs was calculated by the following formula: [(IL-5 concentration induced by OVA) − (IL-5 concentration induced by OVA + CpG ODN level)]/(IL-5 concentration induced by OVA). The 50% inhibitory concentrations (IC₅₀) of the CpG ODNs were calculated from a linear curve of the CpG ODN concentration-percent inhibition.

**Measurement of cytokine levels in culture supernatants.** Murine IL-5, IFN-γ, IL-10, and IL-12 levels were measured with a sandwich enzyme-linked immunosorbent assay kit (OptEIA, Pharmingen) according to the instructions of the manufacturer.

**RESULTS**

**Depletion of NK cells and CD8+ T cells reduces CpG ODN-induced IFN-γ induction but does not prevent suppression of Th2 responses.** CpG ODNs were added at the time of in vitro OVA stimulation of splenocytes isolated from OVA- and alum-immunized C57BL/6 mice and were found to inhibit antigen-induced IL-5 production in a concentration-dependent manner (Fig. 1A). IL-4 was not detectable in the culture supernatants of OVA-stimulated splenocytes from OVA- and alum-immunized C57BL/6 mice by enzyme-linked immunosorbent assay in these studies; by use of splenocytes from BALB/c mice, however, both IL-4 and IL-5 were detectable under the same experimental conditions, and CpG ODNs inhibited IL-4 similarly to IL-5 (data not shown). As we have been using mice with a C57BL/6 background to study the effects of CpG ODNs in murine models of allergic asthma (23, 24) and have a number of cytokine KO mice bred with this background, we used C57BL/6 mice for the remainder of this study and measured IL-5 as a marker for Th2 immune responses. We also found previously that CpG ODNs induce IFN-γ and that this induction is significantly enhanced when CpG ODNs are added with antigen to the splenocytes from antigen-immunized mice (22).

Since NK cells are known to be able to release significant quantities of IFN-γ (10), we hypothesized that the induction of IFN-γ from NK cells by CpG ODNs might promote antigen-specific Th1 responses and consequently inhibit Th2 cytokine production. To test this hypothesis, we evaluated the effects of CpG ODNs on antigen-induced cytokine production from NK cell-depleted splenocytes. As we expected, CpG ODNs induced somewhat less IFN-γ from splenocytes without NK cells than from splenocytes with these cells (Fig. 1B). On the other hand, CpG ODNs inhibited antigen-induced IL-5 production from this cell population, similar to their effects on unfractonated splenocytes (Fig. 1A). There were no differences in the IC₅₀ of the CpG ODNs whether NK cells were present or absent in the culture (Table 1).

Since CD8+ T cells can produce IFN-γ in an antigen-specific manner through major histocompatibility complex class I on antigen-presenting cells (APCs), we speculated that CD8+ T cells might mediate CpG ODN-induced inhibition of IL-5 production. Indeed, we found that splenocytes depleted of CD8+ cells produced significantly less IFN-γ than unfractonated splenocytes did after stimulation with antigen plus CpG ODNs (Fig. 1B). Nevertheless, CpG ODNs inhibited IL-5 production as readily in the absence of CD8+ cells as in the presence of CD8+ T cells (Fig. 1A), and there were no differences in the IC₅₀ of CpG ODNs for splenocytes cultured in the absence of CD8+ T cells and splenocytes cultured in the absence of CD8+ T cells (Table 1). Finally, both NK cells and CD8+ T cells were depleted from our culture system; as expected, stimulation with CpG ODNs resulted in significantly less IFN-γ (Fig. 1B), but the level of inhibition of IL-5 production was essentially unchanged (Fig. 1A) and there were no differences in the IC₅₀ of CpG ODNs (Table 1). These data suggest that although both NK cells and CD8+ T cells affect the Th1 (IFN-γ) response stimulated by CpG ODNs, they do not play an important role in the CpG ODN-mediated inhibition of antigen-induced IL-5 production.

**Effects of IFN-γ and IL-12 on CpG ODN-induced inhibition of IL-5 production.** Having demonstrated the absence of a correlation between the production of IFN-γ and inhibition of IL-5 in association with stimulation by CpG ODNs (Fig. 1A and B and Table 1), we next reconsidered the role of IFN-γ on CpG ODN-induced immunomodulation. In order to clarify these effects in established Th2 immune responses, a neutralizing MAb against IFN-γ (MAb R46A2) was added to the cultured splenocytes from sensitized mice along with CpG ODNs and antigen. Neutralizing MAbs against IL-12 (MAbs
C15.6 and C17.8) were also used since CpG ODN-induced IFN-γ is highly dependent on macrophage-derived IL-12 (10). Anti-IFN-γ MAbs did not affect CpG ODN (1.0 μg/ml)-induced inhibition of IL-5 production (Fig. 2A). As expected, the anti-IL-12 MAbs inhibited CpG ODN (1.0 μg/ml)-enhanced IFN-γ production (Fig. 2B) but had no effect on CpG ODN (1.0 μg/ml)-induced inhibition of IL-5 production (Fig. 2C).

To confirm these results, we also studied the effects of CpG ODNs on antigen-induced IL-5 production from splenocytes from antigen- and alum-immunized IFN-γ/IL-12-dKO mice. In previous in vivo studies, we have shown that CpG ODNs are effective in preventing many of the Th2-mediated attributes of asthma in this mouse model, in which the genes for IFN-γ and IL-12 are disrupted (23). We found that CpG ODNs did inhibit

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TABLE 1. IC_{50}s of CpG ODNs for antigen-induced IL-5 production from splenocytes in the presence or absence of NK and CD8^+ T cells

<table>
<thead>
<tr>
<th>Depleted cells from splenocytes</th>
<th>No. of mice</th>
<th>IC_{50} (μg/ml)</th>
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<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>NK</td>
<td>4</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>CD8^+</td>
<td>6</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>NK and CD8^+</td>
<td>4</td>
<td>0.09 ± 0.01</td>
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^a Mice were treated by intraperitoneal injection of OVA and alum once a week for 3 weeks. One week after the final injection of OVA and alum, splenocytes were isolated from each mouse. NK cells and/or CD8^+ T cells were depleted from the splenocytes with a magnetic cell-sorting system. The supernatants were harvested after 72 h of culture. The supernatants were harvested after 72 h of culture.
antigen-induced IL-5 production in these mice in a concentration-dependent manner (Fig. 3), but this inhibition was about 4.3 times weaker than that for splenocytes from wild-type mice (Table 2).

Effects of TGF-β, IFN-γ, and IL-10 on CpG ODN-induced inhibition of IL-5 production. Although IFN-γ and IL-12 partly mediated CpG ODN-induced inhibition of antigen-induced IL-5 production, the data suggested that CpG ODNs have significant IFN-γ- and IL-12-independent effects on the inhibition of antigen-induced IL-5 production. Therefore, we next examined the effects of the anti-inflammatory cytokines TGF-β and IFN-α/β. A neutralizing MAb or antiserum against TGF-β (1D11) or IFN-α/β was added to the splenocyte culture at the same time that CpG ODNs and antigen were added. Anti-TGF-β MAb did not block CpG ODN-induced inhibition of IL-5 production (inhibition of IL-5 by CpG ODNs versus inhibition of IL-5 by CpG ODNs plus anti-TGF-β with CpG ODNs at 0.1 μg/ml, 57.5% ± 5.7% and 49.7% ± 5.7%, respectively; inhibition of IL-5 by CpG ODNs versus inhibition of IL-5 by CpG ODNs plus anti-TGF-β with CpG ODNs at 1 μg/ml, 47.3% ± 6.8% and 41.1% ± 6.8%, respectively).

FIG. 2. Effects of anti-IFN-γ and anti-IL-12 MAb on CpG ODN-induced inhibition of IL-5 production and IFN-γ production from antigen-stimulated splenocytes from actively sensitized C57BL/6 mice. Mice were treated by intraperitoneal injection of OVA and alum once a week for 3 weeks. One week after the final injection of OVA and alum, splenocytes were isolated from each mouse and cultured in the presence or absence of OVA (100 μg/ml). CpG ODNs (0 to 1.0 μg/ml) were added at the time of OVA restimulation. The supernatants were harvested after 72 h of culture. Each column indicates the mean and standard error of the mean of three independent experiments.

TABLE 2. IC50s of CpG ODNs and percent inhibition of antigen-induced IL-5 production from splenocytes of C57BL/6, IFN-γ/IL-12-dKO, and IL-10 KO mice

| Mouse genotype | No. of mice | IC50 (μg/ml) | % Inhibition by CpG ODNs at:
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<tr>
<td></td>
<td></td>
<td>0.1 μg/ml</td>
<td>1.0 μg/ml</td>
</tr>
<tr>
<td>C57BC/4</td>
<td>11</td>
<td>0.12 ± 0.04</td>
<td>62.0 ± 4.9</td>
</tr>
<tr>
<td>IFN-γ/IL-12 dKO</td>
<td>13</td>
<td>0.52 ± 0.04</td>
<td>16.9 ± 4.6</td>
</tr>
<tr>
<td>IL-10 KO</td>
<td>4</td>
<td>0.36 ± 0.20</td>
<td>50.4 ± 7.1</td>
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* Mice were treated by intraperitoneal injection of OVA and alum once a week for 2 or 3 weeks. One week after the final injection of OVA and alum, splenocytes were isolated from each mouse. The cells were cultured in the presence or absence of OVA (100 μg/ml) and CpG ODNs (0 to 1.0 μg/ml). The supernatants were harvested after 72 h of culture.
μg/ml, 21.8% ± 4.1% and 21.3% ± 4.0%, respectively). Anti-IFN-α/β serum also did not block CpG ODN-induced inhibition of IL-5 production (inhibition of IL-5 by CpG ODNs versus inhibition of IL-5 by CpG ODNs plus anti-IFN-α/β with CpG ODNs at 0.1 μg/ml, 49.7% ± 8.8% and 53.1% ± 22.4%, respectively; inhibition of IL-5 by CpG ODNs versus inhibition of IL-5 by CpG ODNs plus anti-IFN-α/β with CpG ODNs at 1 μg/ml, 10.8% ± 3.1% and 14.2% ± 4.2%, respectively).

Next, we focused on IL-10 since CpG ODNs can induce IL-10 (33, 35) and IL-10 is recognized as an immune-suppressive cytokine (15, 47). We measured the levels of IL-10 in the culture supernatant to clarify whether CpG ODNs induced the cytokine in this in vitro system and found, as expected, that CpG ODNs induced IL-10 in concentration-dependent manner following stimulation with the CpG ODNs at concentrations of 0.1 μg/ml or more (Fig. 4A). There was a clear inverse...
correlation between IL-10 production and IL-5 production from splenocytes in the presence of CpG ODNs and antigen (Fig. 4A, inset). In addition, we found that CpG ODNs induced IL-10 in the absence of IFN-γ and/or IL-12 in studies with splenocytes treated with anti-IFN-γ or anti-IL-12 MAb and splenocytes from IFN-γ/IL-12-dKO mice (data not shown). In order to clarify the role of IL-10 on CpG ODN-induced inhibition of IL-5 production, we examined the effect of CpG ODNs on antigen-stimulated splenocytes harvested from OVA- and alum-immunized IL-10-KO mice. CpG ODNs did inhibit antigen-induced IL-5 production in a concentration-dependent manner (Fig. 4B); however, the level of inhibition by CpG ODNs in splenocytes from the IL-10 KO mice was about three times weaker than that in splenocytes from wild-type mice (Table 2). Moreover, the higher concentration of CpG ODNs (1.0 μg/ml) led to only about 65% inhibition for IL-5 production in IL-10-KO mice, while levels of inhibition of about 90 and 85% were observed at that CpG ODN concentration in wild-type mice and IFN-γ/IL-12-dKO mice, respectively (Table 2).

One explanation for the incomplete blockade of the effects of CpG ODNs in the absence of IL-10 may be that IL-10 inhibits not only Th2 cytokines but also Th1 cytokines (7). On the basis of this explanation, we hypothesized that IL-10 may act as a homeostatic mechanism for the effects of CpG ODNs. In that case, the absence of IL-10 may lead to excessive production of IFN-γ (especially at higher concentrations of CpG ODNs), and the resultant induction of IFN-γ may suppress the antigen-induced IL-5 independently of the effects of IL-10. Supporting this hypothesis, CpG ODNs induced obviously more IL-12 in IL-10-KO mice than in wild-type mice (Fig. 5). In wild-type mice, the higher concentration of CpG ODNs (1.0 μg/ml) induced much less IFN-γ than the 0.1-μg/ml concentration did, suggesting that enhanced IL-10 release (following treatment with 1.0 μg/ml; Fig. 4A) could be inhibiting IFN-γ production. As expected from this hypothesis, splenocytes from IL-10-KO mice still released high levels of IFN-γ following treatment with high concentrations of CpG ODNs (1.0 μg/ml), although there was tendency for the levels to be decreased compared with those achieved with a concentration of 0.1 μg/ml. We next investigated whether CpG ODN-induced IL-10 inhibits IFN-γ production through inhibition of IL-12 production since CpG ODN-induced IL-10 may act as a negative-feedback regulator of CpG ODN-induced IL-12 production (33, 35). IL-12 is produced earlier than IL-5, IL-10, and IFN-γ following addition of CpG ODNs in the presence of antigen (Fig. 6), and indeed, IL-12 levels peak within 6 h of stimulation. As shown above (Fig. 2B), IL-12 is necessary for CpG ODN-induced IFN-γ production in this in vitro system. Following the rise in IL-10 concentrations, IL-12 production was reduced in an IL-10 concentration-dependent manner. Although only a small amount of IL-12 was detected after 72 h of culture of splenocytes from wild-type mice in the presence of antigen and CpG ODNs, the splenocytes from IL-10-KO mice produced large amounts of IL-12 after stimulation with antigen and CpG ODNs (Fig. 5).

Finally, in order to clarify whether inhibition of IL-5 production by CpG ODNs is mediated via both the IFN-γ and IL-12 pathway and the IL-10 pathway, we treated splenocytes from IFN-γ/IL-12-dKO mice with an anti-IL-10 MAb and stimulated them with antigen in the presence of CpG ODNs. Treatment with the anti-IL-10 MAb resulted in the complete recovery of inhibition of IL-5 production caused by low-dose CpG ODNs (0.1 μg/ml), but resulted in only the partial recovery of inhibition of IL-5 induced by higher concentrations of CpG ODNs, suggesting the existence of other, as yet undetermined, pathways through which CpG ODNs may inhibit IL-5 production (Fig. 7).

**DISCUSSION**

It is accepted that immune responses are easily manipulated at their onset, whereas modulation of established responses (such as those seen in patients with chronic allergies) is more challenging. Even in most reported murine studies of CpG ODN therapy of allergic asthma, the treatment has been initiated at the systemic sensitization period (24) or before antigen challenge (8, 39, 41). Recently, other groups (22, 34, 36)
have reported that established eosinophilia in a murine model of asthma is reversed by CpG ODNs. It has been postulated that IL-12 determines the initial immune deviation toward a Th1 response by naïve T cells (20). However, it has not yet been clarified how CpG ODNs suppress established Th2 responses. This report demonstrates that CpG ODN-induced suppression of established Th2 responses is partly mediated by IL-12 and IL-10. Thus, this potentially therapeutic effect may operate both through the de novo induction of Th1 responses and through alternate pathways (potentially the induction of TR1 and Th3 by CpG ODNs), which may also act to reduce Th2 responses.

CpG ODNs induce rapid production of IFN-γ from NK cells, a process which is highly dependent on macrophage-derived IL-12 (10). Dendritic cells are a key player in the induction of initial antigen presentation to naïve T cells; they also produce a large amount of IL-12 following stimulation with CpG ODNs (42). This pathway might be the simplest explanation for the ability of CpG ODNs to induce Th1 immune responses. Since IFN-γ inhibits the proliferation of Th2 cells (31), we initially speculated that IFN-γ produced by NK cells could mediate CpG ODN-induced inhibition of established Th2 immune responses. However, NK cells do not play

FIG. 6. Time course of cytokine production from splenocytes from actively sensitized C57BL/6 mice in the presence of antigen and CpG ODNs. Mice were treated by intraperitoneal injection of OVA and alum once a week for 3 weeks. One week after the final injection of OVA and alum, splenocytes were isolated from each mouse and cultured in the presence of OVA (100 μg/ml) and CpG ODNs (0.1 and 1.0 μg/ml). The supernatants were harvested after 72 h of culture. Each column indicates the mean and standard error of the mean for 4 animals. ○, OVA only; △, OVA plus CpG ODNs at 0.1 μg/ml; ■, OVA plus CpG ODNs at 1 μg/ml.

FIG. 7. Effects of anti-IL-10 MAb on CpG ODN-induced inhibition of IL-5 production from antigen-stimulated splenocytes from actively sensitized IFN-γ/IL-12-dKO mice. Mice were treated by intraperitoneal injection of OVA and alum once a week for 3 weeks. One week after the final injection of OVA and alum, splenocytes were isolated from each mouse and cultured in the presence or absence of OVA (100 μg/ml). CpG ODNs (0.1 and 1.0 μg/ml) and an anti-IL-10 MAb (MAb JES5-16E3; 10 μg/ml) were added at the time of OVA restimulation. The supernatants were harvested after 72 h of culture. Each column indicates the mean and standard error of the mean for five animals.
any role in the CpG ODN-induced immunomodulatory effects in our culture system. Recently, Broide et al. (9) reported that CpG ODNs are still effective for prevention of allergic airway inflammation and hyperresponsiveness in mice pretreated with an antibody to deplete NK cells. Our in vitro data were consistent with their in vivo data since Th2 cytokines, especially IL-5, are key factors in the development of allergic eosinophilic inflammation (21). We have found that CpG ODNs themselves induce IFN-γ, and antigen-specific IFN-γ production is also enhanced by CpG ODNs. It has also been reported that CD8ª T cells secrete more IFN-γ following stimulation by CpG ODNs plus antigen compared with the amount secreted following stimulation by CpG ODNs or antigen alone (42). In order to clarify the effects of the IFN-γ produced by CD8ª cells, we depleted CD8ª cells from splenocytes. CpG ODNs were able to inhibit antigen-induced IL-5 in the absence of CD8ª cells in splenocyte culture. Even when both CD8ª cells and NK cells were depleted, CpG ODNs were effective in blocking IL-5 release, although they were not effective in stimulating IFN-γ production. These data suggest that the IFN-γ produced by NK cells or CD8ª cells is not likely the sole factor responsible for inhibition of established Th2 responses by CpG ODNs.

Although NK and CD8ª cells were clearly not responsible for CpG ODN-mediated suppression of Th2 responses, it still could not be ruled out that newly differentiated antigen-specific Th1 cells played a role. We have reported that IFN-γ induced by CpG ODNs is not the only factor required to alter the initial immune response against a sensitizing antigen (23). However, there is no report in the literature regarding the mechanisms for CpG ODN-induced inhibition of established Th2 responses. To clarify whether CpG ODN-induced inhibition of IL-5 production is mediated by IFN-γ, we first used neutralizing antibodies against IFN-γ and IL-12. Since it has been reported that CpG ODNs induce IFN-γ through IL-12 production, we were not surprised that anti-IL-12 completely inhibited IFN-γ. It is reported that exogenous addition of IL-12 enhanced antigen-induced production of IFN-γ from the peripheral blood mononuclear cells of patients with asthma. Although antigen-induced IL-4 production was inhibited by low concentrations of IL-12 (0.05 to 0.1 ng/ml), higher concentrations of IL-12 (1 to 10 ng/ml) enhanced the release of IL-4 in the same experimental setting (29). In the present study, anti-IL-12 treatment resulted in only moderate recovery of the antigen-induced IL-5 production inhibited by CpG ODNs. Moreover, anti-IFN-γ did not inhibit the effects of CpG ODNs on IL-5 production. To confirm these results, we used IFN-γ/IL-12-dKO mice. The splenocytes of these mice were sensitized by treatment with OVA and alum and produced IL-5 after antigen stimulation. Although CpG ODNs at a concentration of 1.0 μg/ml inhibited IL-5 production, the IC50 was more than four times the value for wild-type mice. This series of data suggest that the production of IFN-γ by CpG ODNs via IL-12 is not the only pathway to the induction of inhibition of established Th2 responses by CpG ODNs. These findings support recent reports that induction of IFN-γ and IL-12 might not be necessary for the inhibition of allergic responses in immunotherapy (25, 30). Indeed, Th1 cells do not always inhibit airway inflammation but can, in some circumstances, exacerbate it (17, 32, 46). It is also known that several functions of CpG ODNs are completely IL-12 or IFN-γ independent (11, 18).

Since CpG ODNs exert a variety of biological effects on APCs, including macrophages, dendritic cells, and B cells, we speculated that blockade of these effects through other induced anti-inflammatory cytokines might eliminate CpG ODN-induced inhibition of Th2 immune responses. Although we previously found that type I IFNs do not mediate the effects of CpG ODNs in the initial immune responses toward Th2 (23), IFN-α can inhibit antigen-induced eosinophil recruitment in the airways of sensitized mice (28). CpG ODNs are powerful inducers of IFN-α (40). TGF-β also inhibits allergen-induced eosinophilia, even though it is not clear whether CpG ODNs induce this cytokine (16). However, it seems less likely that these two cytokines play a critical role in the inhibition of IL-5 production by CpG ODNs, since treatment with neutralizing antibody or antiserum against these cytokines did not result in the recovery of CpG ODN-induced inhibition in the in vitro model used for these studies.

Immunotherapy is thought to be effective through alteration of Th2 to Th1 immune responses against a specific antigen. Recently, induction of IL-10 has been proposed as an important mechanism of immunotherapy (2, 6). IL-10 was initially recognized as an anti-Th1 cytokine since IL-12 production can be strongly inhibited by IL-10 (15). However, it has since been shown that IL-10 has the capacity to modulate Th2 immune responses as well (47). Using Th2 clones, antigen-induced IL-4 and IL-5 production can be completely inhibited by addition of IL-10 in a concentration-dependent manner (14). In the present study, the use of a neutralizing antibody against IL-10 led to the partial recovery of CpG ODN-induced inhibition of IL-5. Here, we demonstrated that CpG ODNs induced IL-10 from antigen- and alum-immunized splenocytes in a concentration-dependent manner. This IL-10 production by CpG ODNs showed a clear correlation with inhibition of IL-5 production. Moreover, a lack of IL-10 reduced the level of CpG ODN-induced IL-5 inhibition, although not as dramatically as we would have predicted. To explain this, we speculated that IL-10 depletion also led to overproduction of IFN-γ, since IL-10 inhibits not only Th2 cytokines but also Th1 cytokines. Indeed, we showed that IL-10 depletion increased the level of CpG ODN-induced IFN-γ production. Since the IFN-γ likely inhibited the production of IL-5, recovery of IL-5 inhibition was lessened in the experiments with IL-10-KO mice. This finding is especially striking since CpG ODNs are widely recognized to be Th1 inducers. In the present study, CpG ODNs certainly induced a Th1 response, but production of excessive amounts of IFN-γ appears to be autoregulated by induction of IL-10. Moreover, we observed that a lack of IL-10 increased the amount of IL-12 released; these data suggest that CpG ODN-induced IL-10 can inhibit IFN-γ production through inhibition of IL-12 production. Finally, we found that inhibition of IL-5 production at least by low concentrations of CpG ODNs, was completely blocked by an anti-IL-10 MAb in IFN-γ/IL-12-dKO mice. These data suggest that IFN-γ–IL-12 and IL-10 do not completely mediate the inhibition of IL-5 production by high concentrations of CpG ODNs. The participation of other regulatory proteins is supported by recent reports showing that CpG ODNs can induce suppressors of cytokine signaling factor types 1 and 3 (13). Another possibility includes...
cell-to-cell contact as an important mechanism for CpG ODN-induced suppression of established Th2 responses, since it is reported that CpG ODNs can change APC functions during antigen processing and presentation (3).

Allergic diseases, including asthma, are more common in industrialized countries than in developing countries (5). Von Mutius et al. (44, 45) reported that there is no relationship between air pollution and the development of asthma in children and have postulated that avoidance of infection during childhood might be a risk factor for atopy; others have reported similar results (37, 38). These data suggest that a bias toward a Th2 response against environmental allergens might become dominant without exposure to viral or prokaryotic agents and, speculatively, their DNA. In the past, allergists used bacterial vaccines made up of heat-killed organisms as stand-alone agents or as adjuvants for allergen immunotherapy (27). These adjuvants certainly contained the CpG motifs in bacterial DNA. Immunotherapy is an alternative treatment for allergen immunotherapy. Chloramphenicol, overproduction of IFN-n, and adsorption of CpG ODNs may prove to be effective adjuvants for bacterial DNA. Immunotherapy is an alternative treatment for allergens certainly contained the CpG motifs in bacterial DNA. Immunotherapy is an alternative treatment for allergens.

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