

Oligodeoxynucleotides Containing CpG Motifs Induce Low Levels of TNF- α in Human B Lymphocytes: Possible Adjuvants for Th1 Responses¹

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Oligodeoxynucleotides containing CpG motifs (CpG-ODN) represent potential adjuvants for specific immunotherapy of type I allergies because they foster Th1-like immune responses. However, previous work has shown that CpG-ODN induce systemically active levels of TNF- α in murine macrophages. The goal of the present study was to evaluate the release of TNF- α in human cells by a CpG-ODN proven to induce Th1 immune responses in cells from atopic individuals and in mice. CpG-ODN induced TNF- α in cells from atopic and healthy individuals. However, the amounts were low, as determined by comparison with commonly used Ags. Intracellular cytokine staining of PBMC revealed that CpG-ODN-induced TNF- α derived exclusively from B lymphocytes. TNF- α contributed to the CpG-ODN-augmented proliferation and Ig synthesis in PBMC, but was not involved in IFN- γ synthesis. In conclusion, our findings indicate that certain CpG-ODN induce low amounts of TNF- α in human B lymphocytes and may therefore be used to modulate Th2-biased immune responses in allergic patients. *The Journal of Immunology*, 2001, 166: 3743–3756.

In atopic individuals, high levels of specific IgE Ab directed against allergens lead to allergic reactions such as rhinitis, asthma, and urticaria. T cell responses to several allergens have been characterized thoroughly, indicating that allergen-specific Th2 lymphocytes play a predominant role in these diseases, since cytokines produced by this T cell subset (IL-4, IL-13) are potent IgE-switching factors (1–4). Specific immunotherapy (SIT)³ proved to be an efficient treatment for patients suffering from type I allergy to airborne allergens (5). During this therapy increasing doses of allergen extracts are administered to sensitized individuals according to standardized protocols. Recently, studies revealed that successful SIT is associated with a modulation of the allergen-specific immune response at the T cell level. This shift to a Th0/Th1-like cytokine pattern was indicated by less production of IL-4 and/or augmentation of IFN- γ (6). The vaccine routinely used for SIT consists of standardized total allergen extracts adsorbed to aluminum hydroxide. However, aluminum hydroxide has been shown to induce Th2-type rather than Th1-type immune responses (7). In case of atopic allergy, the use of vaccine adjuvants fostering Th1-like immune responses could certainly augment the efficacy of the treatment.

Bacterial DNA and synthetic oligodeoxynucleotides containing CpG-motifs (CpG-ODN) have attracted attention because they

acted as Th1-promoting adjuvants in mice (8, 9). Furthermore, CpG-ODN triggered protective and curative Th1 responses in leishmaniasis (10, 11). Treatment of mice suffering from experimentally induced bronchial asthma with CpG-ODN prevented airway eosinophilia and subsequent inflammation, characteristics of allergic asthmatic inflammation (12, 13). In addition to the effects in rodents, CpG-ODN were demonstrated to enhance the immunogenicity of vaccines in monkeys (14, 15). At present, descriptions of immunostimulatory effects of CpG-ODN in humans are still restricted to in vitro studies. CpG-ODN induced proliferation and Ig synthesis in B lymphocytes (16, 17). Furthermore, B cells activated with CpG-ODN secreted IL-6 and IL-10 and increased the expression of the surface costimulatory molecules, MHC class II, CD40, CD54, and CD86 (17, 18). APCs, such as monocytes and dendritic cells, were described to mature and to synthesize IL-12 and IL-18 in response to CpG-ODN, which induced NK cells to secrete increased levels of IFN- γ (19–21). Taken together, these findings support the concept that CpG-ODN represent promising adjuvants for humans when a Th1-like immune response is desired (22). However, when considering ODN containing CpG motifs as possible immunomodulating adjuvants, potential disadvantages and/or harmful effects have to be evaluated. CpG-ODN were shown to cause the sequence-dependent induction of large amounts of TNF- α in murine macrophages (23, 24). Furthermore, CpG-ODN-induced TNF- α caused lethal shock due to liver cell apoptosis (25). The information about the ability of CpG-ODN to induce TNF- α synthesis in human cells is still limited (19, 26).

We have previously reported that a phosphorothioate-modified ODN containing three CpG motifs augmented Th1-type cytokines in PBMC from atopic donors (AD) and reduced the synthesis of IgE Ab (21). Moreover, in a murine model of type I allergy, this CpG-ODN modulated the allergic Th2 response to Bet v 1, the major birch pollen allergen (27). In the present study we evaluated the capacity of this CpG-ODN to induce TNF- α production in human cells, a characteristic that would limit its potential application as adjuvant. We determined which cell types produced TNF- α and investigated the dependence of CpG-ODN-induced proliferation, Ig secretion, and IFN- γ synthesis on TNF- α . Our

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³ Abbreviations used in this paper: SIT, specific immunotherapy; AD, atopic donors; CpG-ODN, oligodeoxynucleotides containing CpG motifs; NAD, nonatopic donors; SAC, *Staphylococcus aureus* Cowan strain I; SN, supernatant.

evaluations may be of importance for the development of Th1-promoting vaccine adjuvants that are both active and safe.

Materials and Methods

Allergic and nonallergic individuals

Patients suffering from birch pollen and/or grass pollen allergy (AD) and individuals without atopic background (NAD) were included in this study. Type I allergy was documented by typical case histories, positive skin prick test, and positive radioallergosorbent test (RAST >3; Pharmacia, Uppsala, Sweden). None of the AD had previously undergone SIT.

Oligodeoxynucleotides and reagents

The phosphorothioate-modified oligomeric sequences were synthesized by MWG (Munich, Germany). The CpG-containing DNA sequence (CpG-ODN) was ATCGACTCTCGAGCGTTCTC. A non-CpG-ODN with CG inverted to GC served as the negative control (ATGCACTCTGCAGGCTTCTC). Each ODN was used at a concentration of 3 μ M throughout the study, a concentration determined in previous studies (21). Endotoxin was measured using the *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, MD). The lower detection limit of the *Limulus* amoebocyte lysate assay in our hands was 0.03 endotoxin units (EU)/ml. No endotoxin could be detected in ODN. The LPS sample (Sigma, St. Louis, MO) used had an activity of 45.5 pg/EU. The following reagents were commercially obtained: polymyxin B (Sigma), tick-borne encephalitis virus vaccine (Baxter, Vienna, Austria), purified protein derivative (Statens Serum Institute, Copenhagen, Denmark), and tetanus toxoid (Calbiochem, La Jolla, CA).

Cell culture and proliferation assays

PBMC were obtained from peripheral blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia). PBMC (2×10^6) were cultured in 200 μ l of Ultra Culture medium (BioWhittaker) supplemented with 2×10^{-5} M 2-ME and 2 mM glutamine in 96-well tissue culture plates (Costar, Cambridge, MA). B lymphocytes were isolated by negative selection from PBMC using magnetic beads (B cell negative isolation kit; Dynal, New York, NY). The kit uses a mixture of mouse mAb specific for human CD2, CD3, CD7, CD14, CD16, and CD56 and subsequently magnetic beads coated with anti-mouse IgG. The obtained cell fractions contained consistently <2% CD3⁺, <1% CD14⁺, and <1% CD16/56⁺ cells as determined by flow cytometry. B cells (2×10^5) were cultured in round-bottom 96-well tissue culture plates (Nuncclone; Nunc, Roskilde, Denmark) in the medium described above. Proliferation was evaluated after 48 h by pulsing the cells with [³H]thymidine (0.5 μ Ci/96 tissue culture well) followed by liquid scintillation analysis. All determinations were performed in duplicate. In inhibition experiments, a neutralizing anti-human TNF- α Ab (5 μ g/ml; PharMingen, San Diego, CA) was added simultaneously with CpG-ODN to the cultures. An isotype-matched Ab (PharMingen) served as the control. The addition of the isotype control Ab did not influence the effects of CpG-ODN. To evaluate Ig production, 4×10^6 PBMC were cultured in 24-well tissue culture plates (Costar) with the CpG-ODN for 8 days. Anti-human TNF- α Ab and the isotype control were added on days 0, 3, and 6.

Detection of cytokines and Ig

Cell-free supernatants (SN) were collected at different time points (4–96 h) and analyzed for TNF- α using ELISA with Endogen Matched Ab Pairs

(Endogen, Woburn, MA) according to the manufacturer's instructions. In brief, 96-well plates (Maxisorp; Nunc) were coated overnight at room temperature with anti-human TNF- α mAb (2 μ g/ml). After saturation, SN and standards (Endogen) were incubated for 1 h at room temperature, and bound TNF- α was detected using the matched biotin-labeled anti-human TNF- α mAb (0.9 μ g/ml PBS containing 0.5% Tween 20 and 4% BSA). After incubation with HRP-conjugated streptavidin (Endogen), the color reaction was developed using tetramethylbenzidine substrate (Endogen). The sensitivity limit was 0.6 pg/ml. IFN- γ ELISA (Cytoscreen; BioSource, Camarillo, CA) and Ig ELISA were performed as described previously (21).

Flow cytometry

PBMC were cultured for 10 h with CpG-ODN, non-CpG-ODN, LPS, *Staphylococcus aureus* Cowan strain I (SAC; Calbiochem), or medium alone in the presence of 1 μ g/ml brefeldin A (Sigma). Surface and intracellular cytokine stainings were performed as previously described (21). PBMC were stained with anti-CD3-PerCP, anti-CD14-FITC, anti-CD19-FITC, and anti-CD56-APC (Becton Dickinson, San Jose, CA). To stain intracellular TNF- α , PE-conjugated anti-TNF- α (PharMingen) was used. The level of background staining was assessed by the use of an isotype-matched PE-conjugated mouse Ig (PharMingen). Cells were analyzed on a FACSCalibur (Becton Dickinson). Data were analyzed with the computer program CellQuest (version 3.2.1; Becton Dickinson). Before statistical analysis, isotype control Ab-positive cells were subtracted from anti-TNF- α -positive cells.

Statistical analysis

Statistical significance of differences was determined by the Wilcoxon signed rank test. Differences were considered statistically significant for $p < 0.05$.

Results

Induction of TNF- α by CpG-ODN

To investigate whether ODN containing CpG motifs increased the synthesis of TNF- α , PBMC from 13 atopic and 13 healthy control subjects were incubated in medium alone or in medium containing 3 μ M CpG-ODN or 3 μ M non-CpG-ODN. In addition, cells from NAD were stimulated with LPS (0.1 ng/ml). Cell culture SN were harvested at several time points (4–96 h) and analyzed using ELISA. However, including AD and NAD, CpG-ODN significantly increased TNF- α production compared with medium alone ($p < 0.001$) or non-CpG-ODN ($p < 0.001$; Table I). The optimal time point to detect TNF- α synthesis in PBMC was 12 h (Table I), since TNF- α levels were decreased at the later time points (data not shown). Background levels of TNF- α production varied considerably among individuals. No significant difference was observed between AD and the healthy control subjects. Polymyxin B, a specific inhibitor of LPS, did not affect CpG-ODN-induced TNF- α production, indicating that TNF- α production was not due to endotoxin contamination (data not shown).

Table I. Induction of TNF- α (pg/ml) by CpG-ODN in PBMC^a

AD	Medium	CpG-ODN	Non-CpG-ODN	NAD	Medium	CpG-ODN	Non-CpG-ODN	LPS
1	<0.6	1.2	<0.6	1	<0.6	0.7	1.7	7.3
2	<0.6	<0.6	<0.6	2	8.2	22.8	9.5	44.8
3	1.1	3.4	1.7	3	<0.6	2.3	1.2	6.9
4	<0.6	9.2	<0.6	4	2.3	5.2	2.6	61.6
5	4.5	16.8	7.6	5	49.5	85	45	>300
6	31.5	10.3	11.2	6	2.7	19.2	3.7	47.9
7	1.6	6.0	1.9	7	2.2	5.1	2.6	61.3
8	9.6	34.9	12.7	8	17	84.9	29.6	>300
9	4.4	10.7	3.8	9	2.0	5.8	4.0	114
10	7.5	50.1	35.7	10	2.9	21.9	3.7	47.9
11	72.7	72.5	56.5	11	1.5	6.8	1.8	13
12	<0.6	25	2.9	12	<0.6	5.3	0.7	27.5
13	2.0	14.5	1.5	13	115	476	142	>300

^a PBMC (1×10^7 /ml) were stimulated with medium, 3 μ M ODN, and LPS (0.1 ng/ml), respectively. SN were harvested after 12 h and assessed for TNF- α by ELISA. Means of duplicates are presented.

Induction of TNF- α by different Ags

To estimate the CpG-ODN-induced amount of TNF- α , PBMC from 16 individuals (8 AD and 8 NAD) were stimulated with the tick-borne encephalitis virus vaccine (250 ng/ml), purified protein derivative (10 μ g/ml), and tetanus toxoid (1 μ g/ml). CpG-ODN were used at the concentration of 3 μ M corresponding to a concentration of 19 μ g/ml. TNF- α levels were assessed by ELISA and are summarized in Table II. Again, the optimal incubation period was 12 h. CpG-ODN-induced TNF- α levels were generally lower than the amounts induced by Ags, which are commonly used in humans.

CpG-ODN-induced TNF- α derives from B lymphocytes, but not from monocytes

To determine the cell type(s) responsible for CpG-ODN-induced TNF- α production, staining of cell surface markers and intracellular cytokines was performed in PBMC. The two-color dot plots depicted in Fig. 1 show that TNF- α secretion in CD19⁺ cells markedly increased when PBMC were stimulated with CpG-ODN compared with non-CpG-ODN or medium alone. SAC, a stimulator of TNF- α in B cells, served as a positive control. For B cells the optimal time point for intracellular TNF- α detection was 10 h, compared with 20 and 30 h (not shown). Fig. 2 shows the pooled flow cytometry results from PBMC obtained from six AD and six NAD. Stimulation with CpG-ODN led to a significant rise in the percentage of TNF- α -producing CD19⁺ lymphocytes. The efficiency of non-CpG-ODN was significantly lower than that of CpG-ODN. Neither monocytes (CD14⁺), T lymphocytes (CD3⁺), nor NK cells (CD56⁺) significantly secreted TNF- α in response to CpG-ODN at 10, 20, or 30 h of incubation. As expected, LPS induced significant TNF- α production in monocytes, whereas B cells were not affected. Thus, B lymphocytes represent the sole cell type secreting TNF- α in response to CpG-ODN.

B lymphocytes respond directly to CpG-ODN by TNF- α synthesis

B cells were negatively selected from peripheral blood of six individuals (three AD and three NAD), and SN were analyzed by ELISA (Table III). Again, the optimal time point was 12 h. Purified B lymphocytes synthesized TNF- α in response to CpG-ODN,

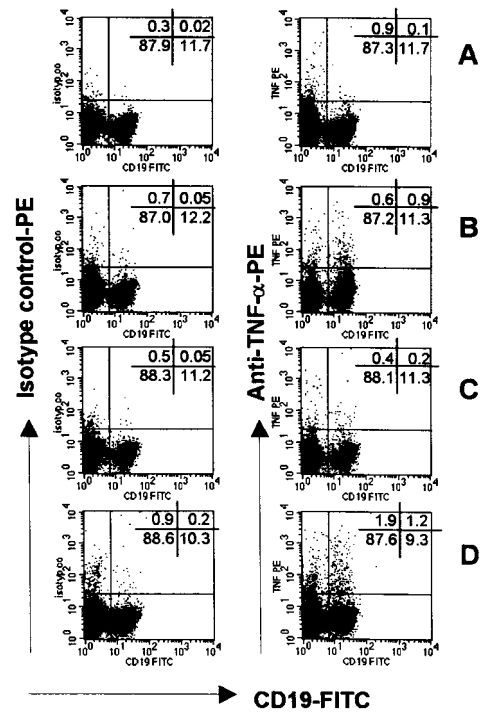


FIGURE 1. B lymphocytes secrete TNF- α in response to CpG-ODN. PBMC were stimulated for 10 h with medium alone (A), CpG-ODN (B), non-CpG-ODN (C), or SAC (D). Thereafter, cells were stained for CD19-FITC and for isotype control-PE or TNF- α -PE. For analysis, a gate was set on lymphocytes by scatter properties. This figure shows one representative two-color flow cytometric analysis of 12 independent experiments. TNF- α secretion in CD19⁺ cells markedly increased in cultures stimulated with CpG-ODN.

as suggested by the experiments with PBMC. Non-CpG-ODN also induced increased amounts of TNF- α compared with medium alone. These data indicate that TNF- α induction by CpG-ODN is a direct effect on B cells without the contribution of other cell types.

Table II. TNF- α (pg/ml) synthesis in PBMC in response to commonly used Ags^a

	Medium	CpG-ODN	Tick-borne Encephalitis Virus	Purified Protein Derivative	Tetanus Toxoid
1	<0.6	1.2	1.7	16.9	<0.6
2	<0.6	<0.6	22.9	108	7.9
3	1.1	3.4	13.5	11.6	ND ^b
4	<0.6	9.2	25.7	>300	1.1
5	1.9	20.5	131	110	1.7
6	1.6	6.1	7.3	75.7	10.8
7	1.7	15.2	12.2	16.2	1.9
8	3.8	5.8	12.7	120	2.9
9	<0.6	0.7	10.5	9.1	ND
10	8.2	22.8	43.1	62.6	8.3
11	<0.6	2.3	9.2	11.2	ND
12	2.3	5.2	38.4	12.6	1.8
13	49.5	85	53	>300	48.7
14	2.7	19.2	22.2	124	ND
15	7.3	7.8	5.3	41.4	4.3
16	4.5	12.9	20.7	19.6	3.2

^a PBMC were cultured for 12 h in medium alone, 3 μ M CpG-ODN (19 μ g/ml), tick-borne encephalitis virus vaccine (250 ng/ml), purified protein derivative (10 μ g/ml), and tetanus toxoid (1 μ g/ml), respectively. SN were harvested after 12 h and assessed for TNF- α by ELISA. Individuals 1–8 were AD and 9–16 were NAD. Means of duplicates are presented.

^b ND, Not done.

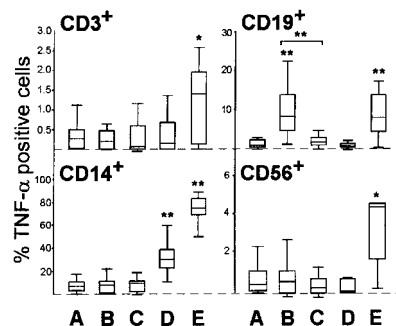


FIGURE 2. CpG-ODN-induced TNF- α derives exclusively from B lymphocytes. PBMC from 12 individuals were cultured in medium alone (A) or with CpG-ODN (B), non-CpG-ODN (C), LPS (0.1 ng/ml; D), or SAC (E) and stained for surface markers as described in *Materials and Methods*. After intracellular cytokine staining, the percentage of TNF- α -producing cells of each cell type was calculated. Isotype control-positive cells were subtracted from anti-TNF- α -positive cells. Box plots of pooled flow cytometry experiments are shown. Each box represents the interquartile range containing the 50% of values. The line across the box indicates the median, and the whiskers extend to the highest and lowest values. CpG-ODN significantly increased the percentage of CD19⁺ TNF- α -secreting cells. This was not observed in T cells (CD3⁺), monocytes (CD14⁺), or NK cells (CD56⁺); by Wilcoxon signed rank test: *, $p < 0.05$; **, $p < 0.01$.

TNF- α is involved in CpG-ODN-induced proliferation and Ig production, but not IFN- γ synthesis, in PBMC

B cells have been shown to proliferate when PBMC were cultured with CpG-ODN (17). Since TNF- α is an autocrine growth factor for B lymphocytes, we investigated whether the mitogenic effects of CpG-ODN on PBMC depended on TNF- α . As shown in Fig. 3A, the addition of a neutralizing anti-human TNF- α Ab significantly inhibited CpG-ODN-triggered proliferation in PBMC derived from nine individuals compared with the isotype-matched control Ab. Moreover, the anti-TNF- α Ab significantly reduced CpG-ODN-augmented synthesis of IgG (Fig. 3C) and IgM (Fig. 3D) in cells derived from five AD. On the other hand, the CpG-ODN-induced synthesis of IFN- γ in PBMC was not significantly affected by the anti-human TNF- α Ab (Fig. 3B).

Discussion

The release of high levels of TNF- α (>100 pg/ml of blood) can cause systemic shock-like reactions in humans (28). It was reported that CpG-DNA is capable to induce TNF- α in a sequence-dependent manner (24). Therefore, we analyzed whether an ODN containing three CpG-motifs, which represents a promising adjuvant candidate for SIT of allergic patients, triggered the synthesis of TNF- α . Here, we show that this phosphorothioate-modified DNA sequence induces TNF- α in PBMC derived from atopic as well as healthy individuals. However, the amounts of TNF- α are relatively low and comparable to the amounts induced by common

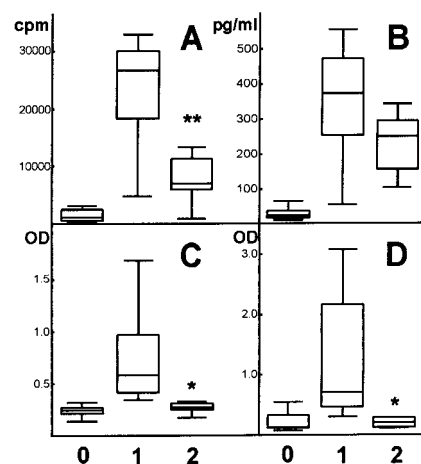


FIGURE 3. TNF- α is involved in CpG-ODN-induced proliferation and Ig, but not in IFN- γ , synthesis. PBMC were incubated in medium alone (0), with CpG-ODN and either an isotype-matched control Ab (1) or a neutralizing anti-human TNF- α Ab (2). Proliferation was determined by [³H]thymidine incorporation assays after 48 h (A); IFN- γ levels were assessed after 24 h (B). Ig synthesis was determined in SN of PBMC incubated with CpG-ODN and the respective Ab (added on days 0, 3, and 6) after 8 days (C and D). The addition of the anti-human TNF- α Ab significantly inhibited proliferative responses (A) and IgG (C) and IgM (D) synthesis (by Wilcoxon signed rank test: *, $p < 0.05$; **, $p < 0.01$). In contrast, IFN- γ production induced by CpG-ODN was not significantly reduced (B).

immunological stimuli, e.g., by Ags routinely used in humans. Our study reveals that CpG-ODN-induced TNF- α derives exclusively from human B lymphocytes and represents a key cytokine involved in CpG-ODN-induced proliferation and Ig secretion in PBMC. The capability of CpG-ODN to induce IFN- γ in PBMC is, however, independent of B cell-derived TNF- α .

The goal of the present study was to evaluate possible harmful cytokine-mediated effects of a CpG-ODN proven to induce Th1 cytokines in human cells and mice (21, 27). We observed that the stimulation of human PBMC with CpG-ODN induced a significant increase in TNF- α production in vitro. This effect was identical in AD and healthy control subjects. However, several indications suggest that the synthesis of TNF- α can be regarded as a phenomenon of less importance: we found that 2 EU/ml LPS, an amount beyond the allowed limit for LPS in parenteralia according to the Pharmacopoeia Europea, elicited markedly higher levels of TNF- α in cells from every individual tested. Furthermore, the comparison with Ags such as tetanus toxoid, purified protein derivative, and the tick-borne encephalitis virus vaccine revealed that these substances stimulated the production of similar levels of endogenous TNF- α . Although it is difficult to extrapolate in vitro data to the situation in vivo, our data provide evidence that this particular DNA sequence will not induce systemically active amounts of TNF- α when administered as vaccine adjuvant.

Table III. CpG-ODN induce TNF- α production in purified B lymphocytes^a

AD	Medium Alone	CpG-ODN	Non-CpG-ODN	SAC	NAD	Medium Alone	CpG-ODN	Non-CpG-ODN	SAC
1	1.2	91.9	3.0	191	1	<0.6	28.4	2.3	96.3
2	<0.6	104.3	3.7	>300	2	<0.6	15.9	<0.6	62.5
3	1.6	54.9	17.7	>300	3	<0.6	51.3	ND ^b	ND ^b

^a B cells were negatively selected from the peripheral blood and cultured (1×10^6 /ml) with medium alone, CpG-ODN, and non-CpG-ODN. SN were obtained after 12 h and TNF- α was analyzed by ELISA. Means of duplicates are presented.

^b ND, Not done.

Recently, Sparwasser et al. (25, 29) suggested a possible role for bacterial DNA and certain synthetic ODN displaying unmethylated CpG motifs in the septic shock syndrome, since certain CpG-ODN induced murine macrophages to release high amounts of TNF- α . Along with T cells, the predominant cell type responsible for TNF- α production in general is the macrophage. It is therefore of importance that, according to our findings, B lymphocytes represented the sole source of CpG-ODN-stimulated TNF- α synthesis in human peripheral blood. In contrast to CD19⁺ cells, monocytes did not significantly accumulate TNF- α in response to CpG-ODN, as determined by intracellular cytokine staining (Fig. 2). Apart from that, these observations provide further evidence that CpG-ODN and LPS substantially differ in their ability to induce immunological effects (26). CpG-ODN stimulated the synthesis of TNF- α in purified B lymphocytes, a fact that was also observed in chronic lymphocytic leukemia B cells (30). Together, these data strongly suggest that CpG-ODN directly induce TNF- α in B cells without the contribution of other cell types or exogenous cytokines. As the control ODN also induced measurable TNF- α synthesis in purified B cells and PBMC, we determined whether the presence of the hexameric palindrome was responsible for these effects. Two additional CpG-ODN (1668, A2) that do not contain a palindrome and their respective controls containing inverted GC motifs were examined (31). The TNF- α levels induced by these CpG-ODN were comparable to those induced by the DNA sequence under investigation in our study (data not shown). The control GC-ODNs also stimulated TNF- α synthesis in some individuals, however, significantly less than the respective CpG-ODN (data not shown). From this we cannot exclude a contribution of the phosphorothioate backbone itself to the observed B cell activation as has been reported previously (16).

Several studies have suggested an important role for TNF- α in the regulation of B cell proliferation and differentiation (32). B lymphocytes have been shown to synthesize TNF- α upon appropriate stimulation, including pharmacologic and natural stimuli, e.g., EBV and HIV (33). ODN containing CpG motifs might trigger TNF- α synthesis in B cells analogous to viral infections. This corroborates the hypothesis that CpG motifs are sensed as danger/infection signals in potentially pathogenetic DNA. Neutralizing anti-TNF- α Ab significantly reduced CpG-ODN-induced proliferation in PBMC, which was mainly attributed to B cells, and the synthesis of IgG and IgM (17). This leads to the speculation that the mitogenic effects of CpG-ODN on B cells are regulated by TNF via an autocrine loop, again in analogy with the behavior of EBV-transformed B cells (34). In addition, SAC-induced proliferation in PBMC could be reduced by the anti-TNF- α Ab, whereas in cultures stimulated with LPS no proliferation was detected (data not shown). On the other hand, TNF- α did not significantly influence CpG-ODN-induced IFN- γ synthesis, which predominantly derives from NK cells (21).

The success of SIT, the treatment of choice in certain forms of type I allergy, can be attributed to a change from Th2 toward Th1 immune responses at the level of allergen-specific T lymphocytes. Recently, allergen-specific T lymphocytes were shown to develop into Th1-like cytokine-secreting effector cells by phosphorothioate ODN (35). We have previously shown that polyclonal IgE production is reduced by CpG-ODN (21). Therefore, the use of CpG-ODN as vaccine adjuvants represents a promising strategy for future SIT (36). However, potential undesired effects, such as the induction of high levels of TNF- α , need to be excluded. We show that CpG-ODN-induced TNF- α derives exclusively from B lymphocytes and can be considered an epiphenomenon.

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References

1. Spiegelberg, H. L., L. Beck, D. D. Stevenson, and G. Y. Ishioka. 1994. Recognition of T cell epitopes and lymphokine secretion by rye grass allergen Lolium perenne I-specific human T cell clones. *J. Immunol.* 152:4706.
2. Ferreira, F., K. Hirtenlehner, A. Jilek, J. Godnik-Cvar, H. Breiteneder, R. Grimm, K. Hoffmann-Sommergruber, O. Scheiner, D. Kraft, M. Breitenbach, et al. 1996. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J. Exp. Med.* 183:599.
3. Bohle, B., H. Schwihla, H. Z. Hu, R. Friedl-Hajek, S. Sowka, F. Ferreira, H. Breiteneder, C. A. Bruijnzeel-Koomen, R. A. de Weger, G. C. Mudde, et al. 1998. Long-lived Th2 clones specific for seasonal and perennial allergens can be detected in blood and skin by their TCR-hypervariable regions. *J. Immunol.* 160:2022.
4. Bohle, B., B. Wagner, U. Vollmann, D. Buck, B. Niggemann, Z. Szepefalusi, G. Fischer, O. Scheiner, H. Breiteneder, and C. Ebner. 2000. Characterization of T cell responses to Hev b 3, an allergen associated with latex allergy in spina bifida patients. *J. Immunol.* 164:4393.
5. Bousquet, J., R. Lockey, and H. J. Malling. 1998. Allergen immunotherapy: therapeutic vaccines for allergic diseases: a WHO position paper. *J. Allergy Clin. Immunol.* 102:558.
6. Durham, S. R., and S. J. Till. 1998. Immunologic changes associated with allergen immunotherapy. *J. Allergy Clin. Immunol.* 102:157.
7. Wiedermann, U., B. Jahn-Schmid, R. Fritsch, L. Bauer, H. Renz, D. Kraft, and C. Ebner. 1998. Effects of adjuvants on the immune response to allergens in a murine model of allergen inhalation: cholera toxin induces a Th1-like response to Bet v 1, the major birch pollen allergen. *Clin. Exp. Immunol.* 111:144.
8. Roman, M., E. Martin-Orozco, J. S. Goodman, M. D. Nguyen, Y. Sato, A. Ronaghy, R. S. Kornbluth, D. D. Richman, D. A. Carson, and E. Raz. 1997. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* 3:849.
9. Chu, R. S., O. S. Targoni, A. M. Krieg, P. V. Lehmann, and C. V. Harding. 1997. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J. Exp. Med.* 186:1623.
10. Zimmermann, S., O. Egeter, S. Hausmann, G. B. Lipford, M. Rocken, H. Wagner, and K. Heeg. 1998. CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J. Immunol.* 160:3627.
11. Walker, P. S., T. Scharf-Kersten, A. M. Krieg, L. Love-Homan, E. D. Rowton, M. C. Udey, and J. C. Vogel. 1999. Immunostimulatory oligodeoxynucleotides promote protective immunity and provide systemic therapy for leishmaniasis via IL-12- and IFN- γ -dependent mechanisms. *Proc. Natl. Acad. Sci. USA* 96:6970.
12. Kline, J. N., T. J. Waldschmidt, T. R. Businga, J. E. Lemish, J. V. Weinstock, P. S. Thorne, and A. M. Krieg. 1998. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* 160:2555.
13. Broide, D., J. Schwarze, H. Tighe, T. Gifford, M. D. Nguyen, S. Malek, J. Van Uden, E. Martin-Orozco, E. W. Gelfand, and E. Raz. 1998. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* 161:7054.
14. Jones, T. R., N. Obaldia III, R. A. Gramzinski, Y. Charoenvit, N. Kolodny, S. Kitov, H. L. Davis, A. M. Krieg, and S. L. Hoffman. 1999. Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenicity of a peptide malaria vaccine in Aotus monkeys. *Vaccine* 17:3065.
15. Davis, H. L., I. I. Suparto, R. R. Weeratna, Jumintarto, D. D. Iskandriati, S. S. Chamzah, A. A. Ma'ruf, C. C. Nente, D. D. Pawitri, A. M. Krieg, et al. 2000. CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. *Vaccine* 18:1920.
16. Liang, H., Y. Nishioka, C. F. Reich, D. S. Pisetsky, and P. E. Lipsky. 1996. Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* 98:1119.
17. Hartmann, G., and A. M. Krieg. 2000. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J. Immunol.* 164:944.
18. Yi, A. K., D. M. Klimman, T. L. Martin, S. Matson, and A. M. Krieg. 1996. Rapid immune activation by CpG motifs in bacterial DNA: systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J. Immunol.* 157:5394.
19. Bauer, M., K. Heeg, H. Wagner, and G. B. Lipford. 1999. DNA activates human immune cells through a CpG sequence-dependent manner. *Immunology* 97:699.
20. Hartmann, G., G. J. Weiner, and A. M. Krieg. 1999. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl. Acad. Sci. USA* 96:9305.
21. Bohle, B., B. Jahn-Schmid, D. Maurer, D. Kraft, and C. Ebner. 1999. Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN- γ production in cells from allergic individuals and inhibit IgE synthesis in vitro. *Eur. J. Immunol.* 29:2344.
22. Heeg, K., and S. Zimmermann. 2000. CpG DNA as a Th1 trigger. *Int. Arch. Allergy Immunol.* 121:87.
23. Halpern, M. D., R. J. Kurlander, and D. S. Pisetsky. 1996. Bacterial DNA induces murine interferon- γ production by stimulation of interleukin-12 and tumor necrosis factor- α . *Cell. Immunol.* 167:72.
24. Lipford, G. B., T. Sparwasser, M. Bauer, S. Zimmermann, E. S. Koch, K. Heeg, and H. Wagner. 1997. Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur. J. Immunol.* 27:3420.

25. Sparwasser, T., T. Miethke, G. Lipford, A. Erdmann, H. Hacker, K. Heeg, and H. Wagner. 1997. Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor- α -mediated shock. *Eur. J. Immunol.* 27:1671.
26. Hartmann, G., and A. M. Krieg. 1999. CpG DNA and LPS induce distinct patterns of activation in human monocytes. *Gene Ther.* 6:893.
27. Jahn-Schmid, B., U. Wiedermann, B. Bohle, A. Repa, D. Kraft, and C. Ebner. 1999. Oligodeoxynucleotides containing CpG motifs modulate the allergic TH2 response of BALB/c mice to Bet v 1, the major birch pollen allergen. *J. Allergy Clin. Immunol.* 104:1015.
28. Michie, H. R., K. R. Manogue, D. R. Spriggs, A. Revhaug, S. O'Dwyer, C. A. Dinarello, A. Cerami, S. M. Wolff, and D. W. Wilmore. 1988. Detection of circulating tumor necrosis factor after endotoxin administration. *N. Engl. J. Med.* 318:1481.
29. Sparwasser, T., T. Miethke, G. Lipford, K. Borschert, H. Hacker, K. Heeg, and H. Wagner. 1997. Bacterial DNA causes septic shock. *Nature* 386:336.
30. Decker, T., F. Schneller, T. Sparwasser, T. Tretter, G. B. Lipford, H. Wagner, and C. Peschel. 2000. Immunostimulatory CpG-oligonucleotides cause proliferation, cytokine production, and an immunogenic phenotype in chronic lymphocytic leukemia B cells. *Blood* 95:999.
31. Klinman, D. M., A.-K. Yi, S. L. Beaucage, J. Conover, and A. Krieg. 1996. CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon- γ . *Proc. Natl. Acad. Sci. USA* 93:2879.
32. Rieckmann, P., J. M. Tuscano, and J. H. Kehrl. 1997. Tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) in B-lymphocyte function. *Methods* 11:128.
33. Sung, S. S., L. K. Jung, J. A. Walters, W. Chen, C. Y. Wang, and S. M. Fu. 1988. Production of tumor necrosis factor/cachectin by human B cell lines and tonsillar B cells. *J. Exp. Med.* 168:1539.
34. Gibbons, D. L., M. Rowe, A. P. Cope, M. Feldmann, and F. M. Brennan. 1994. Lymphotoxin acts as an autocrine growth factor for Epstein-Barr virus-transformed B cells and differentiated Burkitt lymphoma cell lines. *Eur. J. Immunol.* 24:1879.
35. Parronchi, P., F. Brugnolo, F. Annunziato, C. Manuelli, S. Sampognaro, C. Mavilia, S. Romagnani, and E. Maggi. 1999. Phosphorothioate oligodeoxynucleotides promote the in vitro development of human allergen-specific CD4⁺ T cells into Th1 effectors. *J. Immunol.* 163:5946.
36. Van Uden, J., and E. Raz. 1999. Immunostimulatory DNA and applications to allergic disease. *J. Allergy Clin. Immunol.* 104:902.