

## Adjuvant effects of CpG oligodeoxynucleotides on responses against T-independent type 2 antigens

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### SUMMARY

Oligodeoxynucleotides containing CpG motifs (CpG-ODN) are potent *in vitro* B-cell activators and they have been successfully used to increase *in vivo* antibody responses to T-dependent peptide and protein antigens. In contrast, the use of CpG-ODN to enhance *in vivo* antibody responses to various T-independent type 2 (TI-2) antigens has recently generated contradictory results. In this study, we compared the CpG-ODN stimulatory effect on antibody responses of adult and young BALB/c mice to trinitrophenylaminoethyl-carboxymethyl (TNP)-Ficoll and to polysaccharides (PS) from several distinct serotypes of *Streptococcus pneumoniae* (SPn). CpG-ODN co-administration significantly enhanced antigen-specific immunoglobulin M (IgM), IgG, IgG1 and IgG2a titres to TNP-Ficoll. The depletion of CD4<sup>+</sup> cells by monoclonal antibodies (GK1.5) identified their essential role in CpG-ODN-mediated enhancement of antibody responses. In contrast to TNP-Ficoll, CpG-ODN failed to enhance IgM and IgG responses to any of the 18 SPnPS serotypes tested. Providing T-cell epitopes by the conjugation of SPnPS to the carrier protein tetanus toxoid again allowed CpG-ODN to mediate enhancement of IgG, IgG2a and IgG3 responses to most SPnPS serotypes. Thus, antigen-presenting cell/T-cell interaction appears to largely mediate the *in vivo* influence of CpG-ODN on antibody responses to TI-2 antigens. In early life, additional factors limit CpG-ODN modulation of antibody responses to TI-2 antigens.

### INTRODUCTION

Thymus-independent type-2 (TI-2) antigens were originally described as evoking primarily immunoglobulin M (IgM) responses, with little or no IgG antibody formation, and were further defined as capable of inducing antibody responses in T-cell-deficient (*nul/nul*) mice, but not in mice bearing the *xid* mutation or in young mice.<sup>1,2</sup> Among these TI-2 antigens, distinct polysaccharides (PS) constituting the cell wall of

encapsulated bacteria, dextran, or synthetic PS, such as Ficoll, share common features such as large molecular weight, ordered display of multiple identical epitopes and poor biodegradability.<sup>3</sup> Although T cells are not directly primed by TI-2 antigens, a certain regulatory role of T cells and T-cell- or macrophage-derived factors on B-cell responses has been described for most TI-2 antigens, including pneumococcal PS and trinitrophenylaminoethyl-carboxymethyl (TNP)-Ficoll.<sup>3</sup> Recently, mimicking antigen-presenting cell (APC) and T-cell help by co-administration of recombinant interleukin-12 (IL-12) and anti-CD40 monoclonal antibodies (mAb) resulted in a significant increase in antibody titres to pneumococcal PS.<sup>4,5</sup> Furthermore, although Ficoll was initially considered as a model TI-2 antigen, APC and T cells were later shown to control antibody responses to Ficoll.<sup>6,7</sup> Thus, TI-2 antigens represent a rather heterogeneous group of different molecules where the nature of the B-cell activation signal(s) is extremely critical for the determination of both the qualitative and quantitative profiles of immunoglobulin isotype production, probably in response to various cytokines.<sup>3</sup>

Recently, non-methylated CpG-motifs present in bacterial DNA or contained in short synthetic oligodeoxynucleotides (ODN) were shown to induce strong B-cell activation *in vitro*.<sup>8</sup>

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Abbreviations: CpG-ODN, oligodeoxynucleotides containing CpG motifs; ctr-ODN, oligodeoxynucleotides containing no CpG motifs; Pn-23, Pneumovax<sup>®</sup> vaccine containing purified pneumococcal polysaccharides; Pn11-TT, tetanus toxin conjugate pneumococcal polysaccharide vaccine; PS, polysaccharides; TI-2, thymus-independent type 2 antigens; TNP-OVA, trinitrophenyl hapten conjugated to ovalbumin; TNP-Ficoll, trinitrophenylaminoethyl-carboxymethyl-Ficoll; TT, tetanus toxoid; SPnPS, *Streptococcus pneumoniae* serotype-specific polysaccharides.

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Although the exact mechanisms responsible for this B-cell-stimulatory effect are not yet known, they are likely to involve the induction of immediate early genes such as *erg-1*, *c-fos* and *c-myc*.<sup>9</sup> CpG-ODN sequences have also been demonstrated to strongly activate APC and natural killer (NK) cells, to promote the secretion of cytokines such as IL-6, IL-10, IL-12, IL-18, tumour necrosis factor- $\alpha$ , type I interferons, and interferon- $\gamma$  and to induce the expression of major histocompatibility complex and various co-stimulatory molecules.<sup>10</sup> This activation has been shown to result in a strong increase of antibody and T-cell responses to a variety of protein antigens, as reviewed by Wagner.<sup>10</sup> In contrast, controversial results were recently reported from experiments using CpG-ODN to increase antibody responses to TI-2 antigens. In one study CpG-ODN failed to enhance or even suppressed *Pseudomonas aeruginosa*-PS-specific antibody responses,<sup>11</sup> whereas another study reported that CpG-ODN increases TNP-Ficoll specific IgG but not IgM responses.<sup>12</sup> Most recently, CpG-ODN were reported to enhance *Hemophilus influenzae* b (HIB) vaccine responses, but only when HIB-PS were conjugated to a carrier protein.<sup>13</sup> CpG-ODN administration was also shown to enhance antibody responses against two pneumococcal PS conjugated to a diphtheria toxin protein carrier,<sup>14</sup> but responses to plain, unconjugated pneumococcal PS were not evaluated. To understand better the features of CpG-ODN modulation of antibody responses to TI-2 antigens, we assessed the capacity of CpG-ODN to enhance *in vivo* antibody responses to TNP-Ficoll and to a panel of distinct *Streptococcus pneumoniae* (Spn) PS serotypes, which were either administered as plain PS or as protein-conjugated vaccines. Given the potential importance of an enhancement of early-life antibody responses to bacterial PS, and the capacity of CpG-ODN to enhance early-life murine antibody responses to peptide and protein vaccines,<sup>15,16</sup> we also assessed the influence of CpG-ODN on antibody responses elicited by PS immunization in early life.

## MATERIALS AND METHODS

### Mice

Specific pathogen-free adult BALB/c inbred mice were purchased from BRL (Füllinsdorf, Switzerland) and kept under specific pathogen-free conditions. Breeding cages were checked daily for new births. Pups were kept with mothers until weaning at the age of 4 weeks. The evaluation of early-life responses was performed in 2-week-old mice, the earliest age allowing preimmunization bleeding. Mice were bled before and at several time-points after immunization at the tail vein except for mice at 2 weeks of age, which were bled retro-orbitally. Antigen formulations were injected subcutaneously (s.c.) in groups of four to eight mice, unless otherwise indicated in the figure legends. Aliquots of serum from individual mice were assessed either individually or pooled (by group and time-point) for the presence of antigen-specific antibodies.

### Antigens, adjuvants and immunization procedures

Pneumovax-23 [Merck, Sharp and Dohse (MSD), West Point, PA] is a polyvalent pneumococcal vaccine containing 23 different purified cell wall PS from SPn. In our experiments, it was used at a dose of 25  $\mu$ g (20  $\mu$ l) of SPnPS per mouse.<sup>5</sup> Pn11-TT, a polyvalent SPn glycoconjugate vaccine containing 11

different purified cell wall PS from SPn individually conjugated to tetanus toxoid (TT), was kindly provided by Pasteur Mérieux Connaught (Marcy l'Étoile, France). It was used at one-fifth of the human dose (0.1  $\mu$ g to 0.5  $\mu$ g of each SPnPS per dose, and 1  $\mu$ g for serotype 26/6B). Immunizations were performed s.c. at the neck. TNP<sup>45</sup>-Ficoll and TNP<sup>8</sup>-OVA were obtained from Biosearch Technologies (Novato, CA). Both antigens were diluted in saline and each mouse was injected s.c. with a dose of 50  $\mu$ g. In experiments with CD4 depletions, a dose of 25  $\mu$ g TNP-Ficoll was used. Where specified, the antigens were mixed with aluminium hydroxide (AIOH, Chiron, Italy) (0.25 mg for young mice, 1 mg for adult mice) immediately prior to immunization. ODN [CpG-ODN 1826, TCCATGACGTTCTCTGACGTT, or control (ctr)-ODN 1982, TCCAGGACTTCTCTCAGGTT] were used at a dose of 3  $\mu$ g in young mice and 50  $\mu$ g in adult mice, as described previously.<sup>16</sup> ODN with a nuclease-resistant phosphorothioate backbone were provided by the Coley Pharmaceutical Group (Wellesley, MA). Endotoxin levels in all ODN were <0.075 EU/ml tested by Limulus assay (Whittaker Bioproducts, Walkersville, MD). The Na<sup>+</sup> salts of the ODN were ethanol-precipitated and then resuspended in 10 mM Tris (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA) for storage at -20° prior to dilution into phosphate-buffered saline (PBS) for injection. Where indicated, CD4<sup>+</sup> cells were depleted in mice by weekly intraperitoneal (i.p.) injections of 0.3 mg anti-CD4 mAb (GK1.5), starting 1 week before immunization with antigen. The extent of CD4<sup>+</sup> cell depletion was monitored on peripheral blood samples by flow cytometry and was generally >95%.

### Quantification of antigen-specific antibodies

Sera from mice immunized with pneumococcal vaccines, either individual or pooled by experimental groups and time-points, were preadsorbed with Pneumococcal C-polysaccharide (CPS) (Serum Institute Copenhagen, Denmark, 2  $\mu$ g/ml), for 1 hr at room temperature. Sera were then tested by enzyme-linked immunosorbent assay (ELISA) on Nunc Maxisorb plates coated with one of several different pneumococcal serotypes (American Type Culture Collection, Manassas, VA), according to the World Health Organisation Consensus Protocol for determination of specific pneumococcal antibodies, modified for detection of mouse-specific immunoglobulin by use of the relevant isotype-specific (IgM, IgG, IgG1, or IgG2a) peroxidase-conjugated goat or rabbit anti-mouse antibodies (Zymed, San Francisco, CA).<sup>17</sup>

For the detection of TNP-specific antibodies, Nunc Maxisorb plates were coated overnight at 4° with TNP-bovine serum albumin (BSA; Biosearch Technologies, Novato, CA) before saturation with 1% BSA (Sigma, St Louis, MI) in PBS-0.05% Tween-20, for 1 hr at room temperature. Sera were diluted in saturation buffer and serial twofold dilutions (starting at 1/100) were incubated for 2 hr at room temperature. After washing, the relevant isotype-specific peroxidase-conjugated goat or rabbit anti-mouse antibody (Zymed) was added for 2 hr at 37° prior to washing, incubation with substrate and reading. For both tests antibody levels were expressed as ELISA titres by reference to serial dilution of a previously out-titrated serum pool from immunized adult mice. Antibody titres below the cut-off of the assay were given an arbitrary titre of one-half of the cut-off value in order to allow calculation of geometric mean antibody titres.

*Determination of in vitro proliferative responses*

Single spleen cell suspensions were prepared from spleens and cultured at 37° in a 5% CO<sub>2</sub> humidified incubator, in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 1.5 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were seeded on to 96-well plates and treated with media or ODN (at the indicated concentrations) at a density of 10<sup>5</sup> cells/200 µl/well. This procedure was previously shown to induce proliferation of only B cells.<sup>8</sup> At the end of the incubation period, cells were pulsed with 1 µCi of [<sup>3</sup>H]thymidine before harvesting and scintillation counting, as described previously.<sup>8,17</sup> Standard deviations of the triplicate wells were <5%.

*Determination of IgG/IgM titres after in vitro proliferative responses*

For the detection of IgM and IgG in culture supernatants, spleen cells were seeded into 96-well plates and treated with media or ODN (at the indicated concentrations) at a density of 10<sup>6</sup> cells/200 µl/well. After incubation for 48 hr cell supernatants were harvested and immunoglobulin titres determined by ELISA. Nunc Maxisorb plates were coated overnight at 4° with goat anti-mouse IgG Fc (5 µg/ml) or goat anti-mouse IgM (2 µg/ml) in PBS (Cappel/ICN, Costa Mesa, CA), before saturation with 1% BSA (Sigma) in PBS-0.05% Tween-20, for 1 hr at room temperature. Sera were diluted in saturation buffer and twofold serial dilutions of supernatant (starting at 1/2) were incubated overnight at 4°. After washing, peroxidase-conjugated goat anti-mouse IgG or goat anti-mouse IgM (Zymed) were added for 2 hr at 37° prior to washing, followed by incubation with substrate and measurement of optical density (OD). Results of the assays were expressed as ELISA titres by reference to serial dilution of a previously out-titrated serum pool from naïve mice.

*Statistical analysis*

Significance analysis between results obtained from various groups of mice was performed by using the Mann-Whitney U-test. Differences with probability values >0.05 were considered insignificant.

**RESULTS****CpG-ODN enhance *in vivo* antibody responses to TNP-Ficoll**

Adult BALB/c mice (>8-week-old) were first immunized with 50 µg of AIOH-adjuvanted TNP-Ficoll together with either CpG- or ctr-ODN. CpG-ODN administration significantly enhanced peak TNP-specific IgM titres (measured 10 days post-immunization) compared to ctr-ODN (Table 1). CpG-ODN only slightly enhanced total IgG (not shown) and IgG1 titres, but they markedly increased IgG2a antibodies (Table 1). When immunization was performed in 2-week-old mice, CpG-ODN administration significantly enhanced IgG1 but not IgG2a responses to TNP-Ficoll (Table 1). In contrast, when TNP was conjugated to the ovalbumin protein carrier (TNP-OVA), a strong CpG-mediated enhancement of both IgG1 and IgG2a titres was observed regardless of age at immunization (Table 1).

**CpG-ODN fail to enhance antibody responses to plain pneumococcal polysaccharides**

When immunization was performed with 25 µg of a mixture of SPnPS from 23 distinct serotypes (Pn-23), SPnPS IgM responses were detected against serotypes 14, 19/19F, 23/23F and 26/6B (Fig. 1a–d) and against most other serotypes contained in the vaccine (data not shown). As expected, IgM responses peaked 10 days after priming, with no further increase after booster immunization. In contrast to responses to TNP-Ficoll, CpG-ODN had no significant influence on the magnitude or the kinetics of IgM responses against any of 18/23 tested SPnPS (Fig. 1a–d and data not shown). CpG-ODN also failed to enhance IgG, IgG2a and IgG3 responses to plain SPnPS. IgG to all tested serotypes remained at very low titres (<1000 ELISA units), regardless of the administration of CpG- or ctr-ODN (data not shown). Modification of the experimental conditions, including the change of the immunization route (i.p. versus s.c.) or addition of AIOH, failed to result in a CpG-ODN-mediated enhancement of IgM/IgG responses to SPnPS. CpG-ODN also failed to enhance IgM (Fig. 1a–d) or IgG (not shown) responses when immunization was performed at 2 weeks of age.

**Pneumococcal PS do not interfere with CpG-ODN B-cell activation *in vitro***

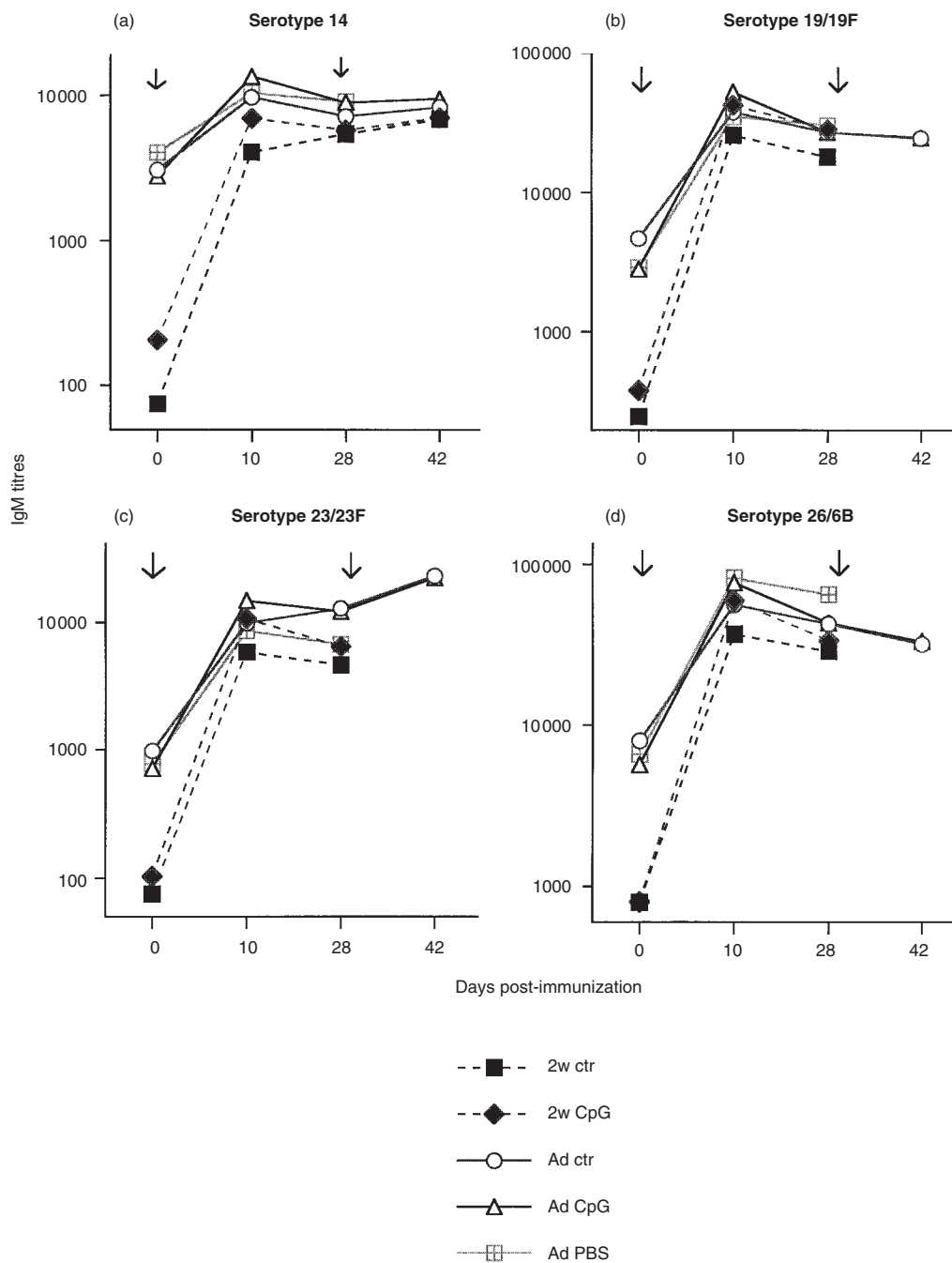
In view of the lack of enhancement of IgM/IgG responses to SPnPS, we asked whether SPnPS could interfere with B-cell activation. Indeed, complex PS taken up by APC are known to be retained for extended periods in the endosomal compartment<sup>18,19</sup> in which CpG-ODN must undergo acidification or other processing steps in order to be able to bind to cytoplasmic CpG-binding proteins and hence deliver their activation signals to the cell nucleus.<sup>10,20</sup> Splenocytes from naïve mice were thus incubated *in vitro* with a mixture of SPnPS and increasing concentrations of CpG- or ctr-ODN, under conditions previously described as leading to B-cell proliferation and polyclonal IgM secretion.<sup>8,21</sup>

As previously observed,<sup>16</sup> B-cell proliferative responses to CpG-ODN stimulation were dose- and age-dependent (Table 2). In spite of lower proliferative responses of cells from 1-week-old mice compared to adult mice, CpG-ODN significantly increased polyclonal IgM production from cells of

**Table 1.** Anti-TNP antibody ELISA titres (log<sub>10</sub>) at 10 days (IgM) or at 28 days (IgG1 and IgG2a) postimmunization with TNP-Ficoll or TNP-OVA. Means and SD of each group

Mice	Antigen	ODN	IgM	IgG1	IgG2a
Ad (n=4)	TNP-Ficoll	CpG-	4.7 ± 0.1*	5.9 ± 0.2	5.5 ± 0.1*
		ctr-	4.5 ± 0.1*	5.4 ± 0.3	3.5 ± 0.1*
2w (n=8)	TNP-Ficoll	CpG-	4.5 ± 0.1	5.1 ± 0.2*	2.8 ± 0.5
		ctr-	4.4 ± 0.1	4.2 ± 0.3*	2.6 ± 0.3
Ad (n=4)	TNP-OVA	CpG-	ND	5.6 ± 0.2¶	5.7 ± 0.1*
		ctr-	ND	4.8 ± 0.5¶	3.1 ± 0.5*
1w (n=8)	TNP-OVA	CpG-	ND	5.4 ± 0.1*	4.0 ± 0.5¶
		ctr-	ND	4.0 ± 0.1*	2.7 ± 0.4¶

\* and ¶ = P < 0.02; ND, not done; Ad, adults; 2w, 2-week-old; 1w, 1-week-old.



**Figure 1.** Lack of influence of CpG-ODN on IgM responses to *S. pneumoniae* polysaccharides (SPnPS). BALB/c mice were immunized with 25  $\mu$ g of SPnPS (Pn-23) together with 50  $\mu$ g (adults: Ad) or 3  $\mu$ g (2 weeks-old: 2w) of CpG-ODN, ctr-ODN or PBS (controls), and boosted 4 weeks later with the same formulation. IgM against SPn serotypes 14 (a), 19/19F (b), 23/23F (c) and 26/6B (d) was measured by ELISA in sera obtained before (day 0) and at several time-points after immunization. Titres are expressed as ELISA units obtained from serum pools of each experimental group containing five to eight mice. Arrows indicate the time of immunization.

all age groups (Table 2). Increasing concentrations of SPnPS (0.1–10  $\mu$ g/ml) remained without inhibitory influence on CpG-ODN mediated B-cell proliferation and IgM production (Table 2). Thus, the B-cell stimulatory activity mediated by CpG-ODN was not inhibited, at least not *in vitro*, by the presence of high concentrations of SPnPS.

#### Conjugation of SPnPS to a protein carrier allows modulation of IgG responses by CpG-ODN, in a serotype- and age-dependent manner

To investigate the role of T-cell epitopes in the CpG-ODN-mediated enhancement of IgG responses to SPnPS, mice were next immunized with a glycoconjugate vaccine containing

**Table 2.** *In vitro* responses of spleen cells from naïve mice of different ages to CpG-ODN

	B cell proliferation* (stimulation index)			IgM production† (end-point titres)		
	1 week	4 week	adults	1 week	4 weeks	adults
Cells alone	1 ×	1 ×	1 ×	1373	3069	7178
CpG-ODN						
3 µg/ml	20 × (11–29)	69 × (38–86)	127 × (89–165)	48 600	16 100	22 950
0.3 µg/ml	11 × (4–13)	45 × (20–54)	61 × (20–104)	29 550	10 189	17 633
0.03 µg/ml	1 × (0–2)	1 × (0–1)	1 × (0–1)	1516	3610	6874
3 µg/ml + Pn-23‡	ND	38 ×	164 ×	ND	12 441	ND
0.3 µg/ml + Pn-23‡	ND	41 ×	105 ×	ND	8362	ND
0 µg/ml + Pn-23‡	ND	1 ×	1 ×	ND	1075	ND
ctr-ODN						
3 µg/ml	3 × (1–6)	3 × (2–4)	3 × (2–3)	3266	4695	7034
0.3 µg/ml	2 × (1–5)	1 × (0–2)	1 × (0–2)	1286	3303	4434
0.03 µg/ml	1 × (0–3)	1 × (0–2)	1 × (0–1)	1222	2933	4466

\*Mean SI from three experiments (adults) to five experiments (1-week- and 4-week-old mice) and the range (in brackets); †Production of non-specific IgM by splenocytes *in vitro*. Results of a representative experiment; ‡Pn-23 was added to *in vitro* cultures in concentrations of 0.1 µg/ml to 10 µg/ml (indicated is 10 µg/ml); different experiment to †; ND, not done.

**Table 3.** Anti-TT antibody ELISA titres (log<sub>10</sub>) at 28 days post-immunization with Pn11-TT. Means and SD of each group

Mice	<i>n</i>	ODN	IgG	IgG1	IgG2a
Ad	(5)	CpG-	5.3 ± 0.1*	5.3 ± 0.1¶	4.3 ± 0.2*
Ad	(5)	ctr-	4.5 ± 0.0*	4.6 ± 0.1¶	2.4 ± 0.2*
2w	(10)	CpG-	4.5 ± 0.2¶	4.5 ± 0.2*	2.8 ± 0.4¶
2w	(8)	ctr-	4.1 ± 0.2¶	4.2 ± 0.2*	2.0 ± 0.0¶

\*and ¶ =  $P < 0.02$ ; Ad, adults; 2w, 2 weeks-old.

11 distinct SPnPS individually conjugated to the TT carrier protein (Pn11-TT). As expected, CpG-ODN strongly enhanced total IgG, IgG1 and IgG2a responses to TT, the protein moiety of the vaccine (Table 3).

In contrast, CpG-ODN remained without any influence on SPnPS-specific IgM responses induced by Pn11-TT (data not shown). CpG-ODN co-administration significantly enhanced IgG responses for four major serotypes (Fig. 2a–d), however, the magnitude of this CpG-ODN-mediated enhancement was modest and serotype-dependent. A significant enhancement of both primary and secondary IgG responses in adult mice was observed for serotypes 14, 19/19F, 23/23F and 56/18C (similar to 23/23F, results not shown), whereas responses to serotype 26/6B were not increased (Fig. 2d). The influence of CpG-ODN on total IgG responses was confirmed by antibody subclass determination, showing an increase of IgG2a (Fig. 3a–d) and of IgG3 antibody responses, which mirrored the IgG2a responses (data not shown). When Pn11-TT immunization was performed in 2-week-old mice, co-administration of CpG-ODN failed to significantly enhance IgG responses to any serotype of SPnPS (Figs 2 and 3), in spite of their enhancement of TT-specific antibodies (Table 3). Thus, addition of CD4 T-cell epitopes to SPnPS allowed CpG-ODN to mediate an enhancement of IgG and IgG2a antibody responses, although only to a modest degree and in a serotype- and age-dependent manner.

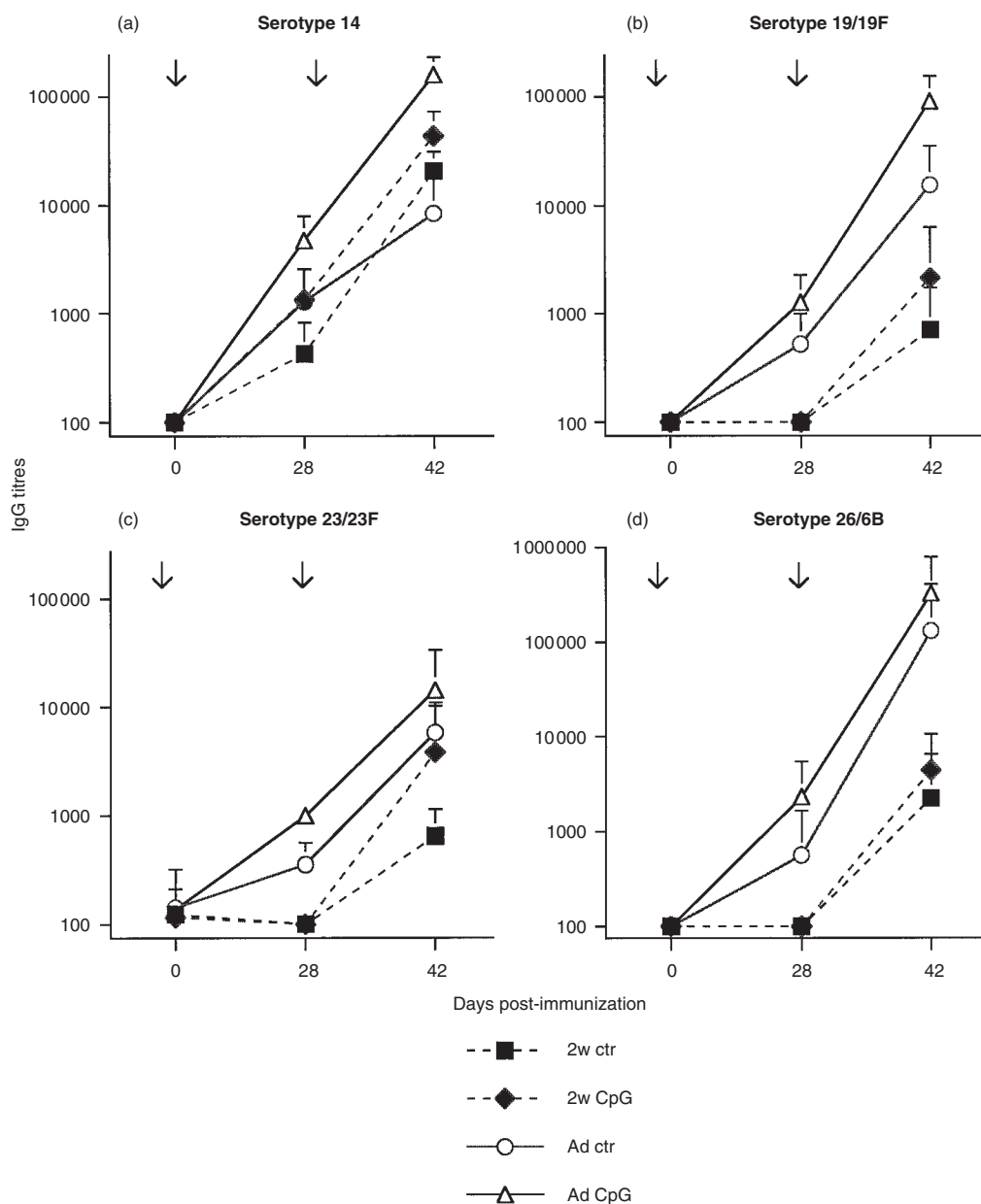
### Role for CD4<sup>+</sup> cells in CpG modulation of antibody responses to TI-2 antigens

The observation of a CpG modulation of antibody responses to TNP-Ficoll and to TT-conjugated SPn but not to unconjugated PS suggested an essential participation for APC/T-cell interaction in the CpG modulation of antibody responses to TI-2 antigens. This was directly assessed in adult mice depleted of CD4<sup>+</sup> cells prior to TNP-Ficoll and CpG-ODN administration. CD4-depletion reduced anti-TNP total IgG measured 3 weeks after immunization in CpG-ODN adjuvanted mice compared to non-depleted mice, such that antibody titres were now similar to those of non-depleted mice immunized with ctr-ODN formulations (Fig. 4). Similar results were obtained for IgG1 titres (Fig. 4). CD4 depletion also reduced IgG2a titres following TNP-Ficoll immunization with CpG-ODN, although IgG2a antibodies remained slightly higher than in mice injected with antigen and ctr-ODN (Fig. 4). Thus, although the depletion of CD4<sup>+</sup> cells did not abrogate IgG, IgG1 and IgG2a responses to alum-adsorbed TNP-Ficoll, the CpG-ODN-mediated enhancement of antibody responses was completely (IgG and IgG1) or largely (83%, IgG2a) dependent on CD4<sup>+</sup> cells.

### DISCUSSION

Our observations indicate that the *in vivo* enhancement of antibody responses to TI-2 antigens by CpG-ODN is dependent on the nature of the PS antigen. B-cell responses to TNP-Ficoll were enhanced by CpG-ODN-mediated immunostimulatory signals, whereas responses to plain SPnPS were not. Our results suggest that these differences might reflect the degree of the participation of other immune cells likely to be modulated by CpG-ODN, such as APC and T cells.

Our observation that CpG-ODN enhance both adult and early-life *in vivo* antibody responses to TNP-Ficoll is in accordance with the recent description of their capacity to enhance the *in vitro* generation of TNP-Ficoll-specific

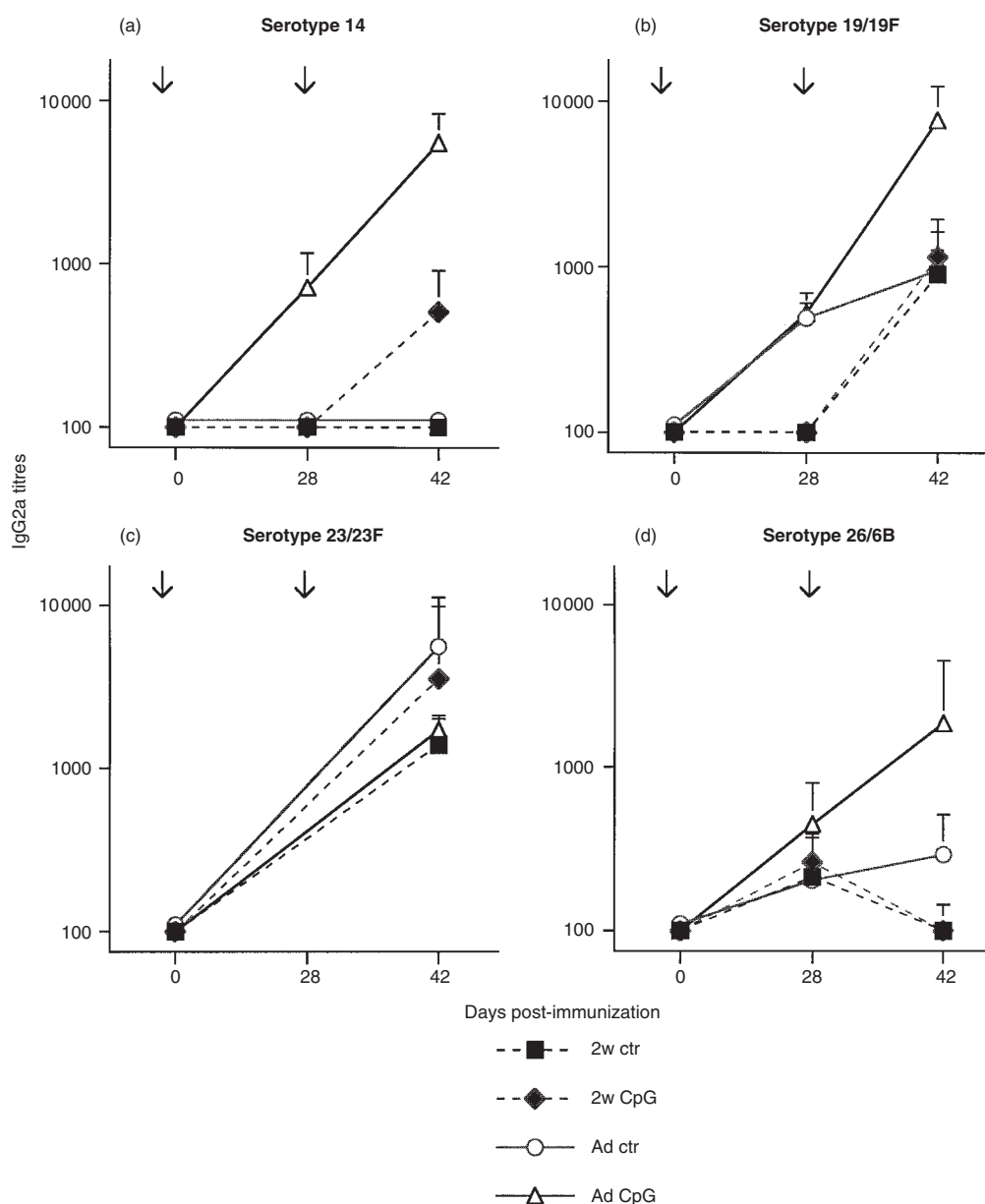


**Figure 2.** CpG-ODN modulation of SPnPS-specific IgG responses to a SPn glycoconjugate vaccine. BALB/c mice were immunized with the glycoconjugate Pn11-TT vaccine together with 50  $\mu$ g (adults: Ad) or 3  $\mu$ g (2 weeks-old: 2w) of CpG-ODN or control-ODN, and boosted 4 weeks later with the same formulation. Total IgG against SPn serotypes 14 (a), 19/19F (b), 23/23F (c) and 26/6B (d) was measured by ELISA in individual sera obtained on day 28 (primary response) and on day 42 (secondary response). Expressed are mean titres and SD in ELISA units of five to ten mice per group. Serotype 14,  $P < 0.02$  adults day 28 and day 42; serotype 19/19F,  $P < 0.02$  adults day 28; serotype 23/23F,  $P < 0.02$  adults day 28 and day 42. Arrows indicate the time of immunization.

antibody-forming cells by adult and neonatal splenocytes.<sup>21</sup> *In vivo*, CpG-ODN are shown here to essentially enhance adult IgG2a responses to TNP-Ficoll, similar to their influence on IgG2a responses to protein vaccines<sup>22,23</sup> and model protein antigens.<sup>24–27</sup> This ‘T helper type 1 driving’ immunomodulatory property of CpG-ODN has been largely associated with their capacity to enhance IL-12 production by APC,<sup>28</sup> which suggests that TNP-Ficoll responses may be influenced by APC activation. In contrast, CpG-ODN failed to increase IgG2a responses in mice primed at 2 weeks of age, despite significant

enhancement of TNP-specific IgG1 antibodies and in spite of their previously reported influence on early-life IgG2a responses to protein antigens.<sup>15,16</sup> Immunization with the T-dependent TNP-OVA formulation confirmed the capacity of CpG-ODN to mediate induction of TNP-specific IgG2a antibodies in early life.

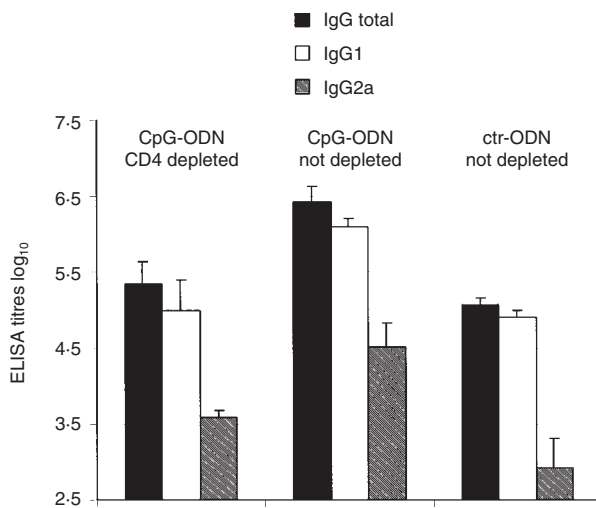
Next, we examined whether CpG-ODN could directly activate Ficoll-specific B cells or if their stimulatory effect was mediated by other immune cells, such as APC or T cells. Responses to TNP-Ficoll have previously been shown to



**Figure 3.** CpG-ODN modulation of SPnPS-specific IgG2a responses to a SPn glycoconjugate vaccine. BALB/c mice were immunized with the glycoconjugate Pn11-TT vaccine together with 50  $\mu$ g (adults: Ad) or 3  $\mu$ g (2 weeks-old: 2w) of CpG-ODN or control-ODN, and boosted 4 weeks later with the same formulation. IgG2a against SPn serotypes 14 (a), 19/19F (b), 23/23F (c) and 26/6B (d) was measured by ELISA in individual sera obtained on day 28 (primary response) and on day 42 (secondary response). Expressed are mean titres and SD in ELISA units of five to ten mice per group. Serotype 14,  $P < 0.02$  adults day 28 and day 42; serotype 19/19F,  $P < 0.02$  day 42; serotype 26/6B, not different due to large variations on day 42. Arrows indicate the time of immunization.

require macrophages, both *in vitro*<sup>6</sup> and *in vivo*.<sup>7,29</sup> The contribution of T cells to anti-Ficoll B-cell responses was also demonstrated by infusing T cells into nude mice.<sup>30</sup> In addition, activated T cells were shown by histology to be co-localized with TNP-specific antibody-secreting cells.<sup>7</sup> To investigate the role of the APC/T-cell interaction in the CpG-mediated influence on TNP-Ficoll responses, CD4<sup>+</sup> cell-depleted mice were immunized with TNP-Ficoll together with CpG- or ctr-ODN. In contrast, although CD4 depletion did not suppress antibody responses to alum-adsorbed

TNP-Ficoll, it almost completely abolished the CpG-ODN-mediated enhancement of antibody responses. Thus, the influence of CpG-ODN on antibody responses to TNP-Ficoll seems largely dependent on the presence of CD4<sup>+</sup> cells. Not only T cells but also certain dendritic cell subpopulations can express CD4<sup>31</sup> and could therefore represent targets for the depleting GK1.5 antibody. We thus conclude that the influence of CpG-ODN on TNP-Ficoll responses is mediated by APC-T-cell interactions, which are abrogated by CD4<sup>+</sup> cell depletion.



**Figure 4.** Depletion of CD4<sup>+</sup> cells abolishes the CpG-ODN-mediated enhancement of IgG responses to TNP-Ficoll. CD4-depleted or non-depleted adult BALB/c mice were immunized with TNP-Ficoll together with either CpG- or ctr-ODN. Three weeks later mice were bled and anti-TNP total IgG, IgG1 and IgG2a titres were measured by ELISA in individual sera. Expressed are mean titres and SD in ELISA units of four or five mice per group.  $P < 0.04$  group CpG-ODN CD4-depleted versus group CpG-ODN not depleted;  $P < 0.03$  group CpG-ODN not depleted versus group ctr-ODN not depleted.

In contrast to Ficoll PS, purified pneumococcal PS induced almost exclusively IgM responses. Although the weak or absent IgG responses to Pn-23 immunization observed in adult BALB/c mice do not correlate to the situation in human adults, where IgG responses can be generated by the same Pn-23 vaccine, they are in accordance with results obtained in human infants as well as in CBA/J mice.<sup>32</sup> Unexpectedly, CpG-ODN co-administration failed to enhance either IgM or IgG responses to unconjugated SPnPS. This failure could have been due to interference of SPnPS on CpG-ODN mediated B-cell activation, but this phenomenon was not observed even when high concentrations of SPnPS were added during *in vitro* incubation of splenocytes with CpG-ODN. Alternatively, CpG-ODN and SPnPS could have drained to distinct sites after s.c. injection, given the preferential localization of PS antigens in the spleen rather than in lymph nodes.<sup>33,34</sup> However, the use of an i.p. immunization route, resulting in preferential draining towards the spleen, also failed to result in a CpG-mediated enhancement of antibody responses to SPnPS. Apart from being different in structure there are also differences in size between TNP-Ficoll and the various SPnPS. Thus, in order to evaluate the influence of CpG-ODN supplementation under optimal conditions for both TI-2 antigens, we used antigen doses formerly reported to induce optimal B-cell responses. Comparison of similar doses (i.e. 25–50 µg) is indeed prevented by inhibition of antibody responses to high doses of SPnPS.<sup>35,36</sup> Interestingly, mixing the pneumococcal vaccine/CpG-ODN combination with AIOH prior to its administration did not significantly alter the immunogenicity of the preparation. This was in contrast to preparations of TNP-Ficoll, TNP-OVA and several other T-dependent antigens,<sup>15,16</sup> where the supplementation with AIOH was required

in order effectively transmit the CpG-ODN-activating effect. These results indicate a fundamental difference in the CpG-ODN-mediated enhancement of T-dependent and TI-2 (SPnPS) immune responses. We hypothesize that the activation of innate immune responses by CpG-DNA evolved primarily as a defence against intracellular pathogens, which commonly lack TI-2 antigens, and that the failure to promote TI-2 responses more effectively may be a mechanism to avoid inducing responses to DNA or other self molecules with repeating epitopes.

In contrast to the lack of influence of CpG-ODN on plain SPnPS responses, it was effective on responses to certain conjugated SPnPS serotypes. To most serotypes, this CpG-ODN-mediated enhancement of IgG responses was of a modest magnitude, but evidenced by the particular enhancement of IgG2a and IgG3 responses. These results are in accordance with a recent report showing that CpG-ODN enhanced antibody responses to the HIB conjugate vaccine, but not to unconjugated HIB-PS.<sup>13</sup> It is interesting to note that the magnitude of the CpG-ODN-mediated enhancement of IgG2a and IgG3 titres was dependent on the pneumococcal serotype. Serotype 14-specific antibody responses were most susceptible to CpG-ODN activation, followed by serotype 23/23F and 56/18C (not shown). In contrast, we observed no enhancement of antibody responses to 26/6B by CpG-ODN administration. Thus, even when additional T-cell epitopes were provided, the capacity of CpG-ODN to enhance adult antibody responses to TI-2 antigens remained dependent on the nature of the SPnPS. This might suggest a distinct modulatory role of APC and/or T cells on B-cell responses to various SPn serotypes.

Unexpectedly, the CpG-mediated enhancement of SPnPS-specific antibody responses to the Pn11-TT glycoconjugate vaccine in adult mice was not observed when in mice immunized at 2 weeks of age. Although we cannot formally exclude that immature APC or T cells are the limiting factors for early-life antibody responses to SPnPS, as described previously for other polysaccharides (Ficoll and Dextran),<sup>37,38</sup> neonatal APC and T cells have been shown to be fully receptive to CpG-ODN, both in previous experiments<sup>15,16</sup> and in this report (TNP-OVA, Table 1). Hence, we hypothesize that a certain immaturity of early-life B cells limits the CpG-ODN modulation of early-life antibody responses to PS.<sup>37,39</sup>

Therefore, it appears that contradictory reports recently issued on the influence of CpG-ODN on antibody responses to PS antigens may be largely explained by the various nature of the PS antigens used and the relative role for APC/T-cell interaction in the modulation of PS-specific B-cell responses. Whether or not CpG-ODN would be able to enhance human adult or early-life responses to plain or conjugated PS can thus not be readily predicted by preclinical studies.

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