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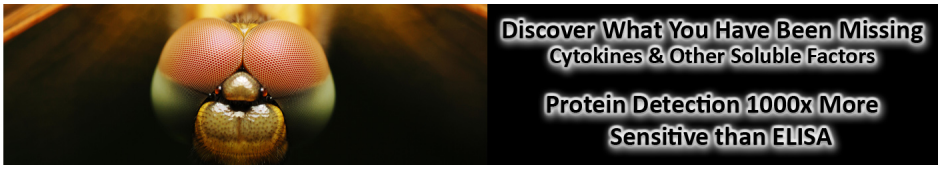

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CpG Oligodeoxynucleotides Can Circumvent the Th2 Polarization of Neonatal Responses to Vaccines But May Fail to Fully Redirect Th2 Responses Established by Neonatal Priming[1]

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Neonatal murine responses to a panel of conventional vaccines differ qualitatively from adult responses by a particular polarization toward a Th2 pattern and a frequent limitation of the Th1 and CTL responses required for protection against intracellular microorganisms. In contrast, DNA vaccines induce adult-like Th1/CTL neonatal responses against the same vaccine Ags. In this report, we show that this can be related to their content in unmethylated CpG motifs. Oligodeoxynucleotides (ODN) containing CpG motifs activate neonatal APCs to produce IL-12 in vitro and induce adult-like Th1 responses to tetanus toxoid and measles Ags in vivo, with production of IgG2a-specific Abs and adult-like secretion of IFN-γ and IL-5 by Ag-specific T cells. However, in spite of their capacity to trigger neonatal B cell proliferation in vitro, CpG-ODN only partially enhanced early life Ab responses. Finally, using Th1-driving CpG-ODN with the boosting dose of a protein vaccine was sufficient to redirect adult but not neonatally primed Th2 responses. These observations could be important for the development of novel vaccines that will have to be effective early in life. *The Journal of Immunology,* **1999, 162: 1611–1617.**

Immunization should be performed very early if one aims to protect young infants from pathogens to which they are already exposed during the first months of life. However, neonates and young infants, as well as newborns from numerous animal species including mice, show particular limitations in generating protective immune responses. Neonatal murine immunization models, using conventional vaccine Ags (measles, tetanus toxoid) in BALB/c mice, were shown under appropriate experimental conditions to depict many similarities with human infant early life responses (1). First, early life B cell responses generally resulted in a slower and weaker increase of vaccine Abs compared with adult mice. They also differed qualitatively from adult responses in that IgM and IgG1 were readily produced, whereas IgG2a, IgG2b, and IgG3 Abs were often reduced in an age-dependent fashion, reflecting the preferential IgM/IgG1 and the lack of IgG2 responses of human infants. Furthermore, analyses of T cell responses to these conventional vaccine Ags indicated that early life T cell differentiation was preferentially polarized toward a Th2 pattern. Although direct evidence is yet lacking, this is also considered as a likely feature following early antigenic exposure in humans (2, 3). This preferential Th2 polarization of early life T cells, observed in a number of murine models (1, 4–7), is currently considered to result from suboptimal interactions between immature APC and T cells (8). As a reflection of this Th2 bias, there is a deficiency in the induction of IFN-γ, TNF-α, and CTL responses that are essential for protection against many intracellular pathogens, and whose induction is thus considered desirable for numerous new vaccines.

In contrast to conventional vaccines, DNA vaccines were recently identified as capable to elicit strong Th1 and CTL responses early in life (9–12). This capacity of DNA vaccines to induce strong neonatal Th1 responses was initially thought to result from the in vivo synthesis of Ag that would result in class I MHC presentation and thereby activation of $CD8^+$ T cells, as well as the prolonged in vivo induction of Ag expression that follows DNA immunization. This Ag expression has been shown in adult mice to last for about 2 wk after the induction of immune responses, at which time Ag-expressing cells are destroyed by CTL (13). Because expression of a nonimmunogenic protein such as luciferase can persist for several months (13, 14), presumably Ag expression could persist in neonates until Th1 and CTL responses were fully developed. However, it was recently found that bacterial DNA and oligonucleotides (ODN)[3] containing unmethylated CpG dinucleotides in particular base contexts (CpG motifs) have direct stimulatory effects on immune responses of adult mice. These stimulatory effects include induction of IFN-$\alpha\beta$, IL-6, IL-12,

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[3] Abbreviations used in this paper: ODN, oligodeoxynucleotides; CpG-ODN, ODNs containing CpG motifs; control-ODN, ODNs containing no CpG motifs; MV, attenuated measles virus; MV-S, measles virus Schwarz strain; HA, hemagglutinin; ALVAC-HA, live recombinant canarypox viral vector expressing the measles virus HA; TTP30, peptide containing the immunodominant epitope of tetanus toxin; AlOH, aluminum hydroxide; HBsAg; hepatitis B virus Ag.

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granulocyte-macrophage-CSF, chemokines, and TNF-α by monocyte/macrophages (15–21), as well as secretion of IFN-γ by NK cells, which all contribute to the subsequent differentiation of T cell responses toward the Th1 subset (20, 22). Accordingly, CpG-ODN, used in coinjection with various Ags, were shown to induce significant Th1-driving adjuvant activity in adult mice (23–30). Whether CpG-ODN would be able to circumvent the high activation requirements of neonatal APC and the weak functional capacity of neonatal NK cells was thus open for study.

In this report, we took advantage of our defined models of neonatal immunizations with various vaccine Ags, the immunodominant tetanus toxoid peptide (TTP30), the attenuated measles virus (MV), and the live recombinant canarypox expressing MV-hemagglutinin (HA) (ALVAC-HA), to evaluate the capacity of a potent Th1-driving CpG-rich ODN (27, 29) to modulate T cell neonatal vaccine responses to Ag presentation systems inducing exclusively Th2 responses (TTP30) or preferential Th2 responses (measles vaccines) in early life. Because part of the adjuvant activity of CpG-rich ODN is attributed to the strong direct stimulation of B cell proliferation and differentiation (16), we asked whether the B cell-promoting effect of CpG-ODN could enhance the weak Ab production capacity of early life. Finally, we asked whether CpG-ODN were capable of redirecting Th2 responses to a protein vaccine established after neonatal or adult priming when only used at booster time.

Materials and Methods

Mice

Specific pathogen-free adult BALB/c inbred mice were purchased from Iffa Credo (L'Arbresle, France) or The Jackson Laboratory (Bar Harbor, ME) and kept under specific pathogen-free conditions. Breeding cages were checked daily for new births, and the day of birth was recorded as the day the litter was found. Pups were kept with mothers until they were weaned at the age of 4 wk.

Vaccines

Live MV Schwarz strain (MV-S; 5×10^5 $CCID_{50}$ per dose), was obtained from Pasteur Mérieux Sérums et Vaccins (Lyon, France). Live recombinant canarypox, expressing the MV-HA (vCp85, ALVAC-HA, 5×10^7 pfu/dose) was obtained from Virogenetics (Troy, NY). TTP30 (1) was used in a dose of 100 μg. Unless otherwise indicated, TTP30 was adsorbed to aluminum hydroxide (AlOH; Sclavo, Siena, Italy), 0.25 mg for young mice and 1 mg for adult mice, immediately before immunization.

Immunization procedures

Mice were immunized i.p. in groups of six to eight. For the experiments with TTP30, the CpG or non-CpG control-ODN were administered at a dose of 100 μg per adult and 20 μg per 1-wk-old mice, to adjust for body weight. For the experiments with the more immunogenic MV-S and ALVAC-HA, the dose of ODN was reduced to 3 μg per young mouse (1 or 2 wk old). The sequence of the CpG-ODN "1826" (27) was TCCAT GACGTTCCTGACGTT and that of the control-ODN "1982" (27) was TCCAGGACTTCTCTCAGGTT. ODN were synthesized with a nuclease resistant phosphorothioate backbone by Oligos Etc. (Wilsonville, OR) or Hybridon (Milford, MA). ODN had undetectable endotoxin levels (less than 1 ng/mg) by *Limulus* assay (Whittaker Bioproducts, Walkersville, MD). The Na^+ salts of the ODN were ethanol precipitated and then resuspended in 10 mM Tris (pH 7.0), 1 mM EDTA for storage at −20°C before dilution into PBS for injection.

Quantification of vaccine-specific Abs

Mice were bled at regular interval for the determination of vaccine-specific serum Abs. Serum MV-HA and TTP30 Abs were measured by ELISA as described (1) using Ag-coated plates or Ltk-HA-transfected cells. Incubation was performed with serial serum dilution starting at 1/100. After washing, the relevant isotype-specific peroxidase-conjugated goat or rabbit anti-mouse Ab (Zymed Laboratories, San Francisco, CA) was added for 2 h at 37°C before washing, incubation with substrate, and reading. Results of MV-HA and TTP30 Abs were expressed by reference to serial dilution of a titrated serum pool from immunized adult mice. Ab titers below the cut off of the assay were given an arbitrary titer of one-half of the cut off value to allow calculation of geometric mean Ab titers.

Determination of T cell responses

Splenocytes were harvested 3 wk after immunization. They were incubated at 37°C with vaccine Ag or in DMEM-10% FCS medium alone (control wells). Cell supernatants were collected after 48 h and 72 h for measurement of IL-5 and IFN-γ content by capture ELISA (1). Values for IL-5 and IFN-γ were expressed by reference to a standard curve constructed by assaying serial dilution of the respective mouse cytokines. Values below the cut off of the assay were given an arbitrary titer of one-half of the cut-off value. Ag-specific cytokine secretion was obtained by subtracting the cytokine content of the supernatant from splenocytes incubated with DMEM alone.

Determination of cellular responses in vitro

Single spleen cell suspensions were prepared and cultured at 37°C in 5% CO_2 humidified incubator in RPMI 1640 supplemented with 10% (v/v) heat inactivated FCS, 1.5 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin, essentially as described (16). Briefly, cells were treated with medium or ODN at the indicated concentrations at a density of 10^6 cells/200 μl/well for experiments to measure induction of cytokine expression or at of 10^5 cells/200 μl/well for proliferation assays. This procedure was previously shown to only induce proliferation of B cells (16). At the end of the incubation, serial dilution of culture supernatants were analyzed for IL-12 and IFN-γ content by ELISA using Abs from PharMingen (San Diego, CA). For proliferation assays, the cells were pulsed with 1 μCi of [^{3}H]thymidine before harvest and scintillation counting (16). SDs of the triplicate wells were <5%.

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 influence on early life B cell responses

To assess the capacity of CpG-ODN to enhance early life Ab responses, we first selected a relatively weak Ag, TTP30. When immunization was performed in 1-wk-old or adult BALB/c mice with TTP30 alone (100 μg resuspended in PBS, in the absence of any adjuvant), even repeated immunization combined with large doses (100 μg in adults, 20 μg in young mice) of CpG-ODN failed to result in a significant induction of anti-TTP30-specific Abs (data not shown). In contrast, with the addition of CpG-ODN together with AlOH to the vaccine formulation, a strong enhancement of Ab responses was observed. In a first set of experiments, adult or 1-wk-old BALB/c mice received a single vaccine dose of AlOH-adjuvanted TTP30 together with either CpG or control-ODN (Fig. 1*a*). The positive influence of CpG-ODN on TTP30 total IgG Abs was already significant 2 wk after priming of adult mice. In contrast, Ab responses triggered in 1-wk-old mice by either CpG or control-ODN remained equally weak for 3 wk after priming. However, between the 3rd and 5th week after priming, a strong increase of TTP30 Ab titers was observed in the absence of any boosting event in the group immunized with TTP30-AlOH plus the CpG-ODN, whereas only a slow and weak Ab response was observed in mice primed with TTP30-AlOH and control-ODN. Thus, the influence of neonatal CpG-ODN addition to TTP30-AlOH only became evident 4 wk after immunization and even then, responses remained weaker than those induced in adults; CpG-ODN increased final Ab titers induced by a single vaccine dose by a factor of 20 when administered to 1-wk-old mice, compared with a 350-fold enhancement in adult mice. When priming was followed by boosting 3 wk after the initial injection (Fig. 1*b*), immunization with the TTP30-AlOH preparation supplemented with CpG-ODN resulted in a strong increase of total anti-TTP30 IgG Abs. In this case, titers were equally high in mice primed at 1 wk of age or as adults and these high levels persisted for at least 3 mo.

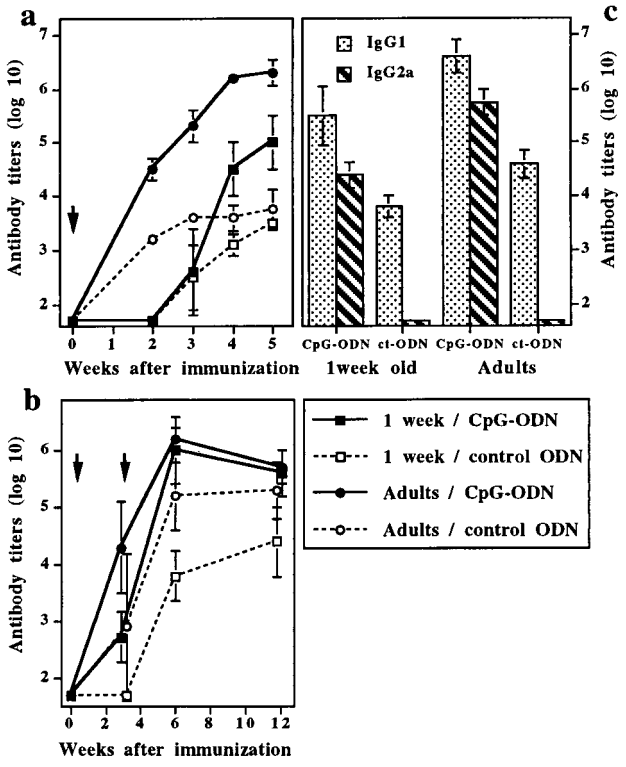

FIGURE 1. Influence of CpG-ODN on Ab response to TTP30 immunization. *a* and *c*, Adult and 1-wk-old BALB/c mice were immunized with one dose only of TTP30-AlOH and CpG-ODN or control-ODN. *a*, Total IgG were measured and expressed as described. *c*, IgG1 and IgG2a Abs were measured at 5 wk postimmunization and expressed as mean vaccine-specific Ab titers (in $\log_{10}$), obtained in groups of four to eight immunized mice (total IgG, 1-wk-old mice CpG-ODN vs 1-wk-old mice control-ODN, $p < 0.01$; total IgG, adult mice CpG-ODN vs adult mice control-ODN, $p < 0.02$; total IgG, 1-wk-old mice CpG-ODN vs adult mice CpG-ODN, $p < 0.01$; IgG1 and IgG2a, 1-wk-old mice CpG-ODN vs adult mice CpG-ODN, $p < 0.02$). *b*, Adult and 1-wk-old BALB/c mice were primed with TTP30-AlOH and CpG-ODN or control-ODN and boosted 3 wk later with the same Ag preparation. TTP30-specific serum IgG Abs were measured by ELISA and expressed as mean vaccine-specific Ab titers (in $\log_{10}$) obtained in groups of four to eight immunized mice.

To define whether the significant differences in the magnitude and the kinetics of Ab responses observed between adult and young mice were related to an intrinsic limitation of early life B cell responses, two distinct sets of experiments were performed. We first evaluated the age at which CpG-ODN administration was capable to rapidly induce Ab production. When TTP30-AlOH-CpG immunization was performed in 4-wk-old mice, Ab production had a significantly earlier onset (reaching 3 $\log_{10}$ 2 wk after immunization) than in 1-wk-old primed mice (<2 $\log_{10}$), although it was still lower than in adults (4.5 $\log_{10}$). This suggested an enhanced-Ab production capacity achieved by 4–5 wk of age that was in accordance with the time at which neonatally triggered responses to TTP30-AlOH became apparent (Fig. 1*a*). The direct influence of CpG-ODN on early life B cells was next assessed in vitro by incubation of spleen cells with CpG or control-ODN, a procedure previously shown to only induce proliferation of B cells (16). When cells from either 1- or 4-wk-old mice were incubated with CpG-ODN for 30 h, a strong proliferation, assessed through [^{3}H]thymidine incorporation at the end of the incubation period, was induced in both age groups, whereas control-ODN remained without detectable influence (Table I). Although lower proliferation indices were observed in 1-wk-old mice, differences were not

Table I. *In vitro responses to CpG-ODN*

	B Cell Proliferation[a] (stimulation index)		IL-12 Production[b] (pg/ml)	
	1 wk	4 wk	1 wk	4 wk
Cells alone	1x	1x	180	0
CpG-ODN				
3 μg/ml	26x	73x	6217	6335
0.3 μg/ml	16x	64x	6007	3453
Ctrl-ODN				
3 μg/ml	2x	5x	234	0
0.3 μg/ml	1x	2x	124	0

[a] Proliferation of B cells from naive mice incubated for 30 h with medium plus CpG- or control-ODN at the indicated concentrations. Proliferation was measured by [^{3}H]Tdr incorporation and is shown as compared with cells from mice from the same age incubated in medium alone.

[b] Production of IL-12 by spleen cells from naive mice incubated for 24 h with medium plus CpG- or control-ODN at the indicated concentrations. IL-12 was measured in the culture supernatant by capture ELISA.

significant. Thus, the limitation of the Ab-producing capacity observed in vivo in early life could not be related to a failure of B cell proliferation in response to CpG-ODN, as assessed under these in vitro experimental conditions.

CpG effects on early life Ab isotype patterns

The influence of CpG-ODN on the pattern of T cell vaccine responses was first addressed through assessing the isotypic distribution of IgG1 and IgG2a vaccine Abs, previously demonstrated as reliable markers of Th2/Th1 responses in this immunization model (1). IgG2a Abs were not detected in mice that received a single administration of TTP30-AlOH with added control-ODN (Fig. 1*c*). In contrast, both adult and 1-wk-old primed mice immunized with TTP30-AlOH-CpG exhibited significant IgG2a vaccine Abs 5 wk after immunization. Similar IgG2a/IgG1 ratios were observed (Fig. 1*c*) whether mice had been immunized once at 1

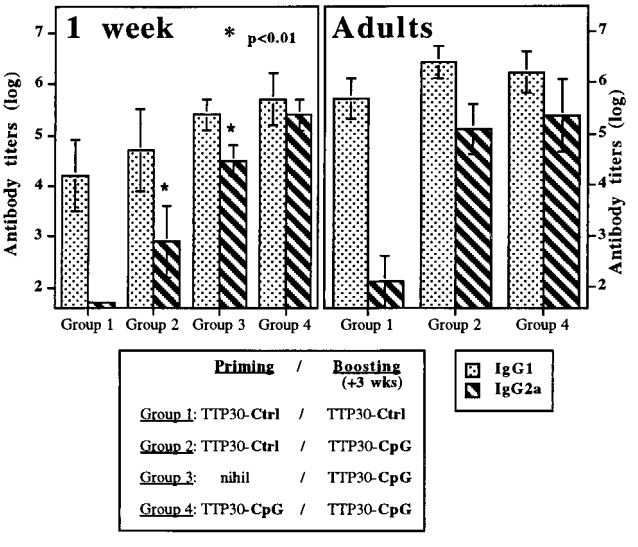

FIGURE 2. Influence of priming on secondary TTP30-specific vaccine responses. Serum IgG1 and IgG2a Ab titers were measured 5 wk after booster immunization of mice that were primed at 1 wk of age or as adults and boosted 3 wk later with various Ag/CpG-ODN combinations, as indicated. TTP30-specific serum IgG1 and IgG2a Abs were measured by ELISA and expressed as mean vaccine-specific Ab titers (in $\log_{10}$) obtained in groups of four to eight immunized mice. (*, IgG2a: group 2 vs group 4, $p < 0.01$).

Table II. *Cytokine levels of supernatants of in vitro restimulated splenocytes*[a]

	Adult Control	1 wk Control	1 wk + CpG	2 wk Control	2 wk + CpG
MV-S					
IFN-γ	340 ± 199	158 ± 69	333 ± 144[b]	456 ± 144	495 ± 94
IL-5	729 ± 168	2621 ± 702	128 ± 61[c]	650 ± 296	<50[c]
ALVAC-HA					
IFN-γ	407 ± 118	94 ± 48	293 ± 93[c]	ND[d]	ND
IL-5	71 ± 93	766 ± 427	106 ± 203[c]	ND	ND

[a] One-week-old and adult Balb/c mice were immunized with MV-S or ALVAC-HA and either CpG-ODN or control-ODN. Splenocytes were harvested 3 wk after immunization and restimulated in vitro with measles Ag. Supernatants were collected after 72 h of culture and their cytokine contents measured by capture ELISA and expressed by reference to standards. Results are expressed as mean of individual cytokine levels (IFN-γ UI/ml; IL-5 pg/ml) ± SD obtained from 4 adult controls − 8 mice per group.

[b] $p < 0.05$ vs. same age controls.

[c] $p < 0.01$ vs same age controls.

[d] ND, not done.

wk, 4 wk (Fig. 2, *left panel*, group 3), or as adults. When a second vaccine dose was given 3 wk after priming, IgG1 and IgG2a Abs reached levels (>5 $\log_{10}$) that were similar in 1-wk-old and adult primed mice. The capacity of CpG-ODN to induce IgG2a Abs in early life was confirmed in another immunization model in which 1-wk-old BALB/c mice were immunized with MV-S in the absence of AlOH; IgG2a Abs remained undetectable after MV-S immunization together with PBS or control-ODN, whereas they reached >3 $\log_{10}$ in mice immunized in the presence of CpG-ODN. These results suggested the capacity of CpG-ODN to induce both Th1 and Th2 differentiation already at 1 wk of age.

CpG effects on early life T cell responses

To directly evaluate the influence of CpG-ODN on early life T cell responses, we selected immunization models that lead to strong T cell responses after a single immunization in early life; this is not readily achieved with the weak TTP30 immunogen. In the first set of experiments, BALB/c mice were immunized once with MV-S in the absence of AlOH with either CpG or control-ODN at the age of 1 wk, 2 wk, or as adults (controls). Three weeks later, Ag-specific cytokine production (IFN-γ and IL-5) was assessed in the supernatants of in vitro restimulated splenocytes (Table II). Splenocytes from 1-wk-old mice, having received control-ODN, secreted lower levels of IFN-γ and significantly higher levels of IL-5 ($p = 0.01$) in response to antigenic restimulation, as compared with adult mice. This confirmed the previously observed Th2 polarization of early life T cell responses to live attenuated MV-S vaccine. Immunization at 1 wk of age in the presence of CpG-ODN significantly enhanced Ag-specific IFN-γ production, restoring it to the same level as that which is generated in adult mice, and had a very strong inhibiting effect on the IL-5 production observed in mice immunized in early life (Table II). Control mice primed at 2 wk of age generated IFN-γ levels similar to adult mice, and these were not further enhanced by addition of CpG-ODN. In contrast, their IL-5 production was reduced to barely detectable levels by CpG-ODN coadministration.

The capacity of CpG-ODN to enhance IFN-γ and to reduce the excessive IL-5 production of neonatally primed T cells was assessed in another immunization model using ALVAC-HA and also given in the absence of AlOH (Ref. 1 and Table II). When 1-wk-old mice were immunized with ALVAC-HA and CpG-ODN, we observed a significant increase of IFN-γ and a complete inhibition of the neonatal IL-5 burst compared with mice coimmunized with the control-ODN (Table II). Levels of both cytokines were indeed similar to those generated by cells from adult controls. This finding suggested the capacity of CpG-ODN to activate neonatal APC so as to support the induction of Th1 responses already in early life. When splenocytes from either 1-wk- or 4-wk-old mice were incubated in vitro for 24 h with either CpG or control-ODN, significant and similar levels of IL-12 were indeed detected in the supernatant of cells from either age group incubated with CpG but not in control-ODN (Table I). High amounts of IFN-γ (>400 pg/ml) were also detected in the supernatant of splenocytes from 1-wk-old mice incubated for 48 h with 3 or 0.3 μg/ml of CpG-, but not control-, ODN (data not shown). Thus, neonatal APC were capable to respond to CpG-ODN stimulation by adult-like IL-12 production and a strong production of IFN-γ.

Influence of CpG on neonatally triggered vaccine responses

We asked whether CpG-ODN, as a potent Th1-driving agent, could successfully redirect neonatally triggered Th2 responses. One-week-old BALB/c mice were primed with TTP30-AlOH in the presence of control-ODN and were boosted 3 wk later in the presence of either CpG- or control-ODN. Other control mice were not immunized at 1 wk of age and only received the injection at 4 wk of age. When mice were primed and boosted with TTP30-AlOH and control-ODN, IgG2a Abs remained below detection levels, reflecting the preferential Th2 polarization previously observed (Fig. 2, *left panel*). When mice primed with TTP30-AlOH and control-ODN were boosted in the presence of CpG-ODN, some IgG2a Abs were induced but they remained at very low levels (3 $\log_{10}$). In fact, IgG2a Abs remained significantly lower than those induced by a single immunization of TTP30-AlOH-CpG given at 4 wk of age (4.5 $\log_{10}$). In contrast, when adult mice were primed with TTP30-AlOH and control-ODN and boosted in presence of CpG-ODN, IgG2a Abs reached high levels (>5 $\log_{10}$) that were similar to those induced by 2 doses of TTP30-AlOH-CpG (Fig. 2, *right panel*). Thus, neonatal priming with control-ODN formulation limited the subsequent induction of TTP30 IgG2a Abs in a persistent manner, whereas TTP30 responses similarly triggered in adults could be fully redirected by the use of CpG-ODN at the time of boost only.

Discussion

This report provides evidence that a single dose of CpG-ODN in neonates is capable of activating APC to produce IL-12 and to trigger adult-like Th1 responses to various vaccine Ags, circumventing the frequent preferential Th2 polarization of early life vaccine responses. However, it identifies two potential limitations of the use of CpG-ODN adjuvants for early life immunization: their relatively weak capacity to enhance primary Ab responses and their failure to fully redirect neonatally triggered Th2 vaccine responses; these factors could limit the benefit of their use at least with certain vaccine Ags.

Evidence that CpG-ODN could induce adult-like Th1/Th2 responses in early life was obtained in three distinct immunization

models characterized by the induction of preferential Th2 responses after early immunization. In the presence of CpG-ODN, Th1-driven IgG2a Abs to TTP30 and to MV-S were induced in mice primed at 1 wk of age. Furthermore, when CpG-ODN were coadministered to 1- or 2-wk-old mice with two types of live viral vaccines that replicate either poorly (MV-S) or not (ALVAC-HA) in mice, T cell responses were characterized by complete suppression of the neonatal IL-5 burst that characterizes the preferential Th2 polarization of neonatal vaccine responses and by the significant increase of IFN-γ. Thus, the Th1-driving capacity of CpG-rich ODN in early life was demonstrated for responses to both live and subunit vaccines.

The in vivo adjuvant effect of CpG-ODN is likely to be related to an effect on APC (17, 20–22, 24, 32–34) and NK cells (35) that promote the subsequent preferential induction of Th1 and CTL responses (20, 27–29, 32). Suboptimal neonatal APC activation is currently considered responsible for the relatively weak generation of Th1/CTL responses in early life, and the existence of distinct activation thresholds for neonatal vs adult APC has been postulated (8). Although progress is being made, the exact molecular mechanisms by which CpG-ODN activate APC are not known. Our direct demonstration that APC from 1-wk-old mice can respond to CpG-ODN with adult-like IL-12 production indicates that all the CpG-related molecular events, including production of reactive oxygen species and NF-κB and certain mitogen-activated protein kinases activation (31, 36–39), can be induced very early in life.

After APC activation and IL-12 production, significant amounts of IFN-γ were detected early (48 h) in the supernatant of neonatal splenocytes incubated with CpG-, but not control-, ODN. Although this has not been formally assessed, this rapid and Ag-independent production of IFN-γ is likely to result from the activation of NK cells (18, 21). The observation that neonatal NK cells can be activated by CpG-ODN could be of significant interest for resistance to pathogens requiring strong innate immune defenses until specific T cell-mediated immunity develops. CpG-ODN were recently shown to protect adult BALB/c mice against *Listeria monocytogenes* (40), and this pathogen is indeed responsible for severe infections in human neonates. Also, the successful induction of neonatal Th1 responses by CpG-activated APC/NK cells will likely enhance associated CTL responses in a number of immunization models, as previously demonstrated in adult mice (28, 29, 41). In the measles model, no direct correlation between Th1/Th2 balance and CTL induction was observed because 1-wk-old mice could generate CTLs to MV-S in spite of limited Ag-specific IFN-γ and high IL-5 responses compared with adults (42). This was also observed by others (43). In contrast, CTL responses to recombinant hepatitis B virus Ag (HBsAg), an Ag that self-assembles into virus-like particles, are only generated if a Th1 environment is provided (29). As expected, CTL responses were observed in mice immunized at 1 wk of age with HBsAg+alum+CpG but not with HBsAg+alum (C. L. Brazolot Millan, et al., unpublished observations). Thus, the addition of CpG-ODN could benefit vaccine Ags that induce preferential Th2 neonatal/adult responses and that benefit from a stronger Th1 environment for enhanced-CTL induction.

The influence of CpG-ODN on Ab production follows both direct B cell activation and enhancement of T cell help (27–29, 31). When the influence of CpG-ODN on Ab response was studied in vivo with a weakly immunogenic peptide (TTP30) in the absence of AlOH, repeated immunization with large doses of CpG-ODN failed to enhance Ab responses either in young or in adult mice. This result is in contrast to coimmunizations with more immunogenic soluble proteins in which the addition of CpG-ODN alone resulted in a significant adjuvant effect in adult mice (28, 29). However, even when used with the stronger HBsAg immunogen, CpG administration also remained ineffective in 1-wk-old mice in the absence of AlOH (data not shown). Thus, in spite of their strong adjuvant potential, CpG-ODN may not be able to transform any weak Ag into highly immunogenic products, particularly in early life. However, TTP30 adsorption to AlOH was sufficient to allow CpG-ODN to strongly enhance TTP30-specific IgG Abs in both young and adult mice. This is likely due to the depot effect of alum, which probably retains the Ag/CpG formulation. However, this depot effect is unlikely to last for several weeks, i.e., until young mice reach immunological maturity, because CpG-ODN-containing formulations only had significant Th1 influence on the vaccine responses of mice immunized at 7 but not at 3 days of age (data not shown). This finding indicates that although alum doubtless has some depot function, it must be fairly short lived. The intrinsic influence of CpG-ODN on early life Ab responses was also demonstrated using measles vaccines that are given without AlOH and are unlikely to persist in vivo given their restricted replication patterns. The fact that otherwise undetectable MV-S IgG2a Abs reached >3 $\log_{10}$ after a single MV-S immunization with CpG-ODN indicates an early influence of CpG on neonatal Ab responses. Thus, when CpG-ODN are given with protein+alum vaccines, they probably capitalize on the depot effect of alum. Indeed, induction of IgG2a Abs to TTP30 is only achieved with the addition of CpG-ODN, even in immunologically mature adult mice.

However, very obvious differences persisted between Ab responses triggered at 1 wk of age and as adults, both in terms of kinetics and magnitude of Ab production. First, early life Ab responses to TTP30-AlOH after immunization with CpG-ODN only became apparent 4 wk postimmunization, i.e., at 5 wk of age; whereas, 2 wk were sufficient in adult mice. Second, although final TTP30 Ab titers raised in CpG-adjuvanted 1-wk-old mice were higher that those of adult controls, they remained at least 10-fold inferior to those of adults receiving CpG-ODN. As a result of this persisting immaturity, adult-like TTP30 Ab levels were only achieved in early life after the administration of a second vaccine dose at 4 wk of age, i.e., at a time of enhanced-B cell maturation.

It has been shown previously that $>95\%$ unprimed adult B cells, exposed in vitro to large doses of CpG-ODN, enter the cell cycle and begin to release IL-6, which leads to their polyclonal activation and IgM secretion (16). We show here that similarly strong proliferative responses could be elicited by CpG-ODN in vitro at 1 or at 4 wk of age. The relative discrepancy between the in vitro responses and suboptimal in vivo early life B cell responses could have several explanations. First, the polyclonal activation observed in vitro could occur in vivo and interfere with the induction of Ag-specific Ab responses. However, under the described experimental conditions, CpG-ODN failed to enhance total IgM Abs upon immunization of either adult or 1-wk-old mice (data not shown), as would have been expected in the presence of strong polyclonal activation (44). Second, early life B cells could be activated by CpG-ODN and induced to proliferate, but remain unable to terminally differentiate into Ab-producing plasmocytes before the age of 4 wk. The description of a 4-wk requirement for the maturation of specific tyrosine kinases in B cells (45) is consistent with this hypothesis. Alternatively, signaling through the B cell receptor that is required for synergy with CpG-mediated activation could remain suboptimal in early life B cells (16). Finally, yet suboptimal APC/T cell/B cell interactions could explain the slower and weaker in vivo IgG Ab responses observed in early life, but the

demonstration of adult-like neonatal APC and T cell cytokine production in response to CpG-ODN does not directly support this hypothesis either.

In addition to the weak capacity of CpG-ODN to rapidly enhance early life Ab responses, another potential limitation to their use in early life immunization programs was identified as their relative failure to redirect neonatally triggered Th2 vaccine responses when CpG-ODN were added only to the booster dose of TTP30+AlOH. We observed that a single immunization with TTP30-AlOH-CpG at 4 wk of age induced significantly higher IgG2a Ab responses than a booster immunization with the same vaccine in mice neonatally primed with TTP30-AlOH and control-ODN. In contrast, CpG-ODN did successfully redirect Th2 responses triggered by TTP30+AlOH in adults, as previously observed in adult mice after priming with hen egg lysozyme Ag in CFA (27). This result indicates that once a Th2 response is established by neonatal priming, it may be more difficult to redirect it to a Th1 response than after adult priming, even when boosting is performed with Th1-driving formulations as strong as CpG-ODN. This is in accordance with our previous observations using the same peptide with a water-in-oil squalene adjuvant formulation (46), as well as with experiments using a canarypox recombinant (ALVAC-HA) and measles DNA vaccines at time of priming and boosting, respectively (9). Interestingly, parallel experiments suggest that CpG-ODN may be able to better redirect neonatally triggered TH2 responses when used with a stronger immunogen (C. L. Brazolot Millan et al., unpublished observations). The weaker vaccine responses frequently observed in early life could thus favor the persistence of the TH2 pattern of neonatally induced responses to some vaccine Ags, as compared with the situation in adults. Whatever the exact mechanisms, this indicates the importance of the immunogenicity of the vaccine formulation used for neonatal priming.

Could CpG-ODN represent the strong but safe adjuvants required for early life immunization? In comparison to DNA vaccines, the use of CpG-ODN as adjuvants would avoid the risk of chromosomal integration. However, their potent and dose-dependent immune stimulation capacity could prove toxic if the doses are excessive. CpG-ODN administration to adult mice at very high doses resulted in toxicity and death (47). Even relatively low doses of CpG caused toxicity by releasing high quantities of TNF-α in galactosamine-sensitized mice (48) and by priming for the Schwartzmann reaction (18). This could be an important issue in early life because repeated vaccine doses are often required to induce sufficient immune responses. The capacity of neonates to tolerate excessive immune activation could also be lower than later in life. Accordingly, although the use of CpG-ODN was generally free of local or systemic toxicity, we have observed an increased morbidity and mortality of 1-wk-old mice administered 20 μg of CpG-ODN with certain live viral vaccines (data not shown). The theoretical risk of eliciting autoimmune Th1-mediated reactions through the use of CpG-ODN (49, 50), in contrast, should not be greater in the more Th2-prone neonatal period than in adult life. Given the interesting properties of CpG-ODN for the induction of Th1/CTL responses in early life, further studies assessing their characteristics and long term safety appear important.

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