CpG-Induced Th1-Type Response in the Downmodulation of Early Development of Allergy and Inhibition of B7 Expression on T Cells of Newborn Mice

Cyro A. de Brito · Ana E. Fusaro · Jefferson R. Victor ·
Paula O. Rigato · Adriana L. Goldoni ·
Bruno P. Muniz · Alberto J. S. Duarte · Maria N. Sato

Abstract

Introduction Several differences have been described between neonatal and adult immune responses. The predisposition in early life to Th2-type response or tolerance makes it a susceptible period for infections and allergic sensitization.

Objective The aim of this work was to evaluate the effects of CpG-containing oligodeoxynucleotides on neonatal and adult immunization with ovalbumin and *Blomia tropicalis* extract and compare the CpG effects on B and T cells of neonatal and adult mice.

Results and Discussion Mice that received CpG showed reduced immunoglobulin E (IgE) antibody production in both neonatal and adult periods, in parallel to increased IgG2a antibody levels. We observed that spleen cells of mice that received CpG in early life produced increased amounts of interferon-γ upon anti-CD3 stimulation. Negative regulation of IgE response was more pronounced in adult than neonate mice; further, CpG decreased anaphylactic antiovalbumin IgG1 only in adults. Also, an upregulation of toll-like receptor 9 expression was detected in adult B cells, but not in neonatal, upon CpG stimuli. Neonatal B cells showed enhanced interleukin (IL)-10 expression and decreased IL-6 levels than adult B cells in response to CpG. When we analyzed in vitro activation of CD4+ T cells, an increased expression of B7 molecules on T cells in neonates was suppressed by CpG.

Conclusion Altogether, we verified qualitative and quantitative evidences regarding CpG effect on neonatal and adult allergens immunizations, which points to the importance of understanding neonatal immune system to establish immunomodulatory strategies for prevention of allergic diseases.

Keywords Oligodeoxynucleotide · neonate · immunomodulation · CpG · costimulatory molecules

C. A. de Brito · A. E. Fusaro · J. R. Victor · P. O. Rigato ·
A. L. Goldoni · B. P. Muniz · A. J. S. Duarte · M. N. Sato (✉)
Laboratory of Investigation in Dermatology and
Immunodeficiencies, School of Medicine,
University of São Paulo,
LIM 56,
São Paulo, Brazil
e-mail: marisato@usp.br

Introduction

In the last decades, it has become evident that allergy development occurs during the first few months of life. Further, changes in lifestyle and diet in childhood may affect the development of atopic disorders [1]. Type I hypersensitivity is characterized by a Th2-dominant response, increased serum immunoglobulin E (IgE) antibody (Ab) levels, and symptoms such as rhinitis, dermatitis, and generally asthma, the prevalence of which increases 50% every decade [2].

Newborn mice have a predisposition to Th2-biased response, making them vulnerable to allergen sensitization. Indeed, allergen sensitization in newborn mice promotes a Th2-dominant memory response when re-exposed with the same allergen in adult life [3]. Th2-biased immune response in neonates involves several factors, such as interleukin (IL)-4-induced apoptosis through IL-13R1 binding, which is highly expressed in newborn Th1 cells [4], and hypomethylation of the conserved noncoding sequence 1 region, a key regulator of Th2 cytokines production in naïve CD4+ T cells [5]. Nonetheless, studies using

particular immunization protocols demonstrate a preserved ability of neonatal T cells to generate robust Th1 responses [6]. Using *Chlamydia trachomatis* infection as a model, neonates were able to develop strong Th1 response and promote efficient chlamydial clearance [7]. Additionally, vaccination of newborn mice with Bacillus Calmett-Guérin was shown to inhibit airway hyperresponsiveness and induce predominant Th1 cytokine responses [8].

Bacterial DNA and oligodeoxynucleotides containing CpG motifs (CpG-ODN) have been described as potent inductors of Th1 cytokines production [9]. CpGs, complexes of dinucleotide cytosine–guanines (CG) bound by phosphor backbones, are present in the unmethylated form in the DNA of bacteria, viruses, and retroviruses, and their concentration is 20-fold higher in microbial than eukaryotic DNA [10]. Immune stimulation by CpG begins with its recognition by the toll-like receptor 9 (TLR-9) that induces a signaling process which, in turn, involves molecules such as MyD88, IRAK, and TRAF-6 and results in mitogen-activated protein kinases and nuclear factor κB activation [11, 12]. Although only some cell populations, such as B cells and plasmacytoid dendritic cells, express TLR-9, CpG-ODN modulates immune responses directly and indirectly by secreted cytokines. In antigen-presenting cells, CpG-DNA induces expression of costimulatory molecules and secretion of cytokines IL-2, interferon (IFN)-α, IFN-γ, IL-6, and IL-12 [13] and immunoglobulins [14]. Activation of B cells through TLR-9 leads to *t-bet* expression and regulates immunoglobulin class switching [15], whereas B cells of TLR-9- or MyD88- deficient mice are unable, both in vitro and in vivo, to produce isotypes dependent on Th1 cytokines after CpG stimulation [16, 17]. Although several works showed that CpG can enhance the Th1 responses of neonates to immunization with tetanus toxoid, measles antigen, and hepatitis B surface antigens [18, 19], some works suggest defective activation of neonatal and cord blood cells by CpG [20, 21]. Further, little is known about the differences in the effects of CpG between newborns and adults.

In the present work, we show the effect of CpG in newborn and adult mice immunization with ovalbumin and dust mite *Blomia tropicalis* extract on antibody and cytokine production. We observed a pronounced and sustained inhibition of IgE Ab production by CpG even in secondary allergen exposure in adult mice. The lower efficacy in CpG modulation in neonatal allergen sensitization suggests that some aspects of the newborn immune system, such as impaired expression of TLR-9 by B cells, may affect innate responses, particularly to pathogen associated molecular patterns (PAMPs) as CpG. Interestingly, we also showed that there is a higher expression of B7 molecules in antigen-stimulated newborn CD4+ T cells than in their adult counterparts, and that CpG was able to

reverse this upregulation of B7 molecules. Although CpG treatment in neonatal period may be less effective than in adult mice, this work points to the importance of understanding neonatal immune system to establish immunomodulatory strategies capable of potentiating innate and adaptive responses for prevention of allergic diseases.

Materials and Methods

Allergens and Oligodeoxynucleotides

OVA (grade V, Sigma, St. Louis, MO, USA) and extract of dust mite *B. tropicalis* (Bt, 37,650 units of biological equivalent per milliliter, kindly provided by International Pharmaceutical Immunology/ASAC, Spain) were used for animal immunization. CpG and control oligodeoxynucleotides with phosphorothioate backbones, 1826 (TCC ATG ACG TTC CTG ACG TT, 5′–3′) and 1745 (TCC ATG AGC TTC CTG AGT CT), were synthesized by Eurogentec (Herstal, Belgium).

Animals

A/Sn mice (3 days old; 8–10 weeks old) and DO11.10 transgenic mice (10 days old; 8–10 weeks old) of both sexes were provided by animal facilities of the School of Medicine and Biomedical Sciences Institute of the University of São Paulo. Wistar Furth rats of both sexes, 3–4 months old and bred from our own laboratory's animal facilities, were used for passive cutaneous anaphylaxis reaction analysis. All experiments were approved by the Ethics Committee for Animal Research of Biomedical Sciences Institute.

Experimental Protocols

Neonatal Immunization Protocols were performed as established and described earlier [22]; A/Sn mice were sensitized by intraperitoneal (i.p.) injections with 10 μg of OVA in 0.6 mg of Al(OH)$_3$ at the ages of 3 and 13 days. Twenty days later, the mice were i.p. boosted with 10 μg of OVA. A similar immunization protocol was used for Bt (40 μg) followed by two i.p. boosts of 150 μg of Bt with an interval of 7 days. Mice were bled 7 days after boost. Groups of mice immunized with OVA or Bt received 4 μg of CpG-ODN (ODN 1826) or control-ODN (ODN 1745) at priming and first boost. Concentrations of ODNs were based on the work of Kovarik et al. [23].

Adult Immunization Eight-to-ten-week-old female A/Sn mice were immunized subcutaneously with 20 μg of allergen (Bt and/or OVA) in 6.0 mg of Al(OH)$_3$. After

2 weeks, mice were boosted by i.p. route with the respective antigen (20 μg of Bt and/or OVA), and an additional boost of Bt immunization was done 7 days later (100 μg of Bt or Bt and OVA). Groups of mice received 50 g of CpG-ODN (ODN 1826) or control-ODN (ODN 1745) at the priming. All mice groups were bled 7 days after the last boost.

Passive Cutaneous Anaphylaxis Reaction

Sera were quantified for anti-OVA and anti-Bt IgE antibody levels by passive cutaneous anaphylaxis (PCA) reaction in rats, as described by Mota and Wong [24]. Individual serum sample dilutions were intradermally injected on the shaved back of the rats. After 18 h, the rats received an injection of either 0.5 mg OVA or 0.25 mg of Bt in 1.0 ml (0.5%) Evans Blue solution through a tail vein. The anaphylactic activity of IgG1 was evaluated by PCA reaction in mice as previously described [25]. Mice have been previously shaved and received intradermal injections of serial dilutions of sera in pool, previously treated for 1 h at 56°C for heat inactivation of IgE. After 2 h, they were intravenously challenged with 0.25 mg OVA in 0.5 ml (0.25%) Evans Blue solution. All tests were conducted in triplicate. PCA titers were expressed as the reciprocal of the highest dilution resulting in a spot bigger than 5 mm in diameter.

Determination of Antibody Levels

Anti-OVA and anti-Bt antibody levels and total IgE levels were measured by means of enzyme-linked immunosorbent assay (ELISA), as previously described [26, 27]. Briefly, 96-well plates (A2, High-binding, Costar, Cambridge, MA, USA) were coated with OVA (5 μg/ml) or Bt (20 μg/ml) or anti-IgE (3 μg/ml) in 0.1 M carbonate bicarbonate buffer (pH 9.5) and incubated for 1 h at 37ºC followed by 18 h at 4ºC. After washing with phosphate-buffered (PBS), plates were blocked with PBS–1% bovine serum albumin (BSA—Sigma, St Louis, Mo, USA) and washed and incubated with serum dilutions for 1 h at 37ºC and for 18 h at 4ºC. After new washes with PBS containing 0.05% Tween™ 20, plates were incubated with biotinylated goat antimouse IgG1, IgG2a, or IgE (Southern Biotechnology Associates, Birmingham, AL, USA) followed by streptavidin peroxidase conjugate (Sigma). Enzymatic activity was detected by the addition of 3,3′,5,5′-tetramethylbenzidine (Sigma) for 15 min at room temperature. Results were expressed as antibody titers (log) obtained by interpolating a titrated serum pool from immunized mice with high levels of anti-OVA or anti-Bt antibodies. Total IgE concentration was correlated with a standard curve from purified immunoglobulin (Pharmingen).

In Vitro Cytokine Production

Mice splenocytes were isolated and cultured in Roswell Memorial Park Institute medium with 10% fetal bovine serum (HyClone III, UT, USA) in 24-well microplates (Costar) under stimulus of OVA (200 μg/ml), anti-CD3 monoclonal antibody (mAb; PharMingen, 1 μg/ml), or lipopolysaccharide (LPS, Sigma, 50 μg/ml) at 37°C in a humidified CO_2 incubator. Splenocytes of DO11.10 transgenic mice were stimulated with 100 μg/ml OVA and CpG-ODN or control-ODN (0.1, 1.0, and 10.0 μg/ml). Supernatants were harvested after 24, 48, and 72 h for cytokine level measurements. The cytokines IFN-γ, IL-4, IL-10, and IL-6 were quantified by enzymatic immunoassay with their respective antibody pairs following manufacturer's instructions (OptEIA, PharMingen). The detection limits were as follows: IFN-γ (7.8 pg/ml), IL-4 (3.2 pg/ml), IL-10 (125 pg/ml), and IL-6 (5 pg/ml).

Flow Cytometry Analysis

Splenocytes of nonimmunized DO11.10 mice or splenic B cells of nonimmunized neonate and adult mice isolated by negative selection using a magnetic labeling kit (Miltenyi Biotec, Bergish Gladbach, Germany) with purity levels of >90% assessed by flow cytometry were cultured with OVA and CpG and harvested after 24, 48, and 72 h. Next, cells were washed with PBS containing 1% BSA and stained with one of the following before fixation with 4% formaldehyde (Sigma): anti-CD4 and anti-CD45R PerCP; anti-CD44 fluorescein isothiocyanate; anti-inducible costimulatory (ICOS), anti-CD80, and anti-CD86 PE (PharMingen). B cells gated by anti-CD45R staining were permeabilized by the addition of 0.5% saponin (Sigma), and intracellular TLR-9 expression was detected with anti-TLR-9 PE (Imgenex, San Diego, CA, USA). Respective isotype controls were used in all analysis. Fluorescence data of 10,000 events were acquired with Epics XL (Beckman Coulter, Fullerton, CA, USA) and then analyzed with Summit v3.3 software (Dako Cytomation, Fort Collins, CO, USA).

Real-Time PCR Analysis

Total RNA was extracted from purified and stimulated B cells using QIAmp RNA blood kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. cDNA synthesis was performed using the *Sensiscript Reverse Transcriptase* kit (Qiagen).

PCR was run with 1.5 μl cDNA sample in 18.5 μl of *Platinum SYBR® Green qPCR SuperMix-UDG* (Invitrogen, Carlsbad, CA, USA) containing 1 μl of *ROX Reference Dye* and 0.2 mM of each primer. Primers for IFN-γ (5′-CAT

GGC TGT TTC TGG CTG TTA CTG-3′; 5′GCC AGT TCC TCC AGA TAT CCA AGA-3′), TLR-9 (5′-TCA TGG ACG GGA ACT GCT ACT ACA-3′; 5′-TCA GAG ACA GAT GGG TGA GAT TGC-3′), IL-10 (5′-AAC AAA GGA CCA GCT GGA CAA CAT-3′; 5′-CTG GAT CAT TTC CGA TAA GGC TTG-3′) and the housekeeping gene β-actin (5′- GCC TTC CTT CTT GGG TAT GGA ATC-3′; 5′-ACG GAT GTC AAC GTC ACA CTT CAT-3′) were synthesized by Invitrogen.

Samples were incubated for 10 min at 95°C and 45 cycles of 15 s/95°C, 30 s/60°C, and 30 s/72°C, in the iCycler (Bio-Rad, USA). Data were analyzed using the iCycler iQ Program software (Bio-Rad). To correct for variations in RNA recovery and the reverse transcription yield, the amount of IFN-γ cDNA was divided by the amount of β-actin cDNA. Results were expressed as fold increases of normalized values, over the levels of untreated cells.

Statistical Analysis

Comparisons between groups were carried out with the following nonparametric tests: Mann–Whitney to compare two independent groups, Wilcoxon test for two paired samples, and Kruskal–Wallis test followed by Dunn's posttest to compare three or more unpaired groups. Differences between groups were considered significant when p values were ≤0.05.

Results

CpG-ODN-Induced Decreasement of IgE Response Is Less Pronounced in Neonatal Immunization with OVA

Murine neonates have typically Th2-biased immune responses [18], mainly in secondary responses [3]. To assess the modulatory effect of synthetic CpG-ODNs on the development of type I hypersensitivity response in early life, 3-day-old A/Sn mice were immunized with OVA and CpG and compared to adult immunization. Figure 1a shows that CpG-ODN treatment significantly decreases anti-OVA IgE antibody production, both in neonate and adult immunized mice. The decrease of IgE response by CpG in neonates was less pronounced than adults. In addition, the levels of IgE in CpG-treated newborn mice were similar to those observed in control-ODN-treated newborn mice, suggesting a nonspecific effect of oligonucleotides in newborn mice. Although control-ODN only induces a nonspecific effect in neonates, the inhibitory effect on the IgE response was more pronounced with CpG treatment. In mice, there are two types of IgG1 Ab, one displaying anaphylactic activity, which is positively regulated by IL-4, and another that lacks this activity and whose synthesis is stimulated by IL-12 or IFN-γ [23]. Therefore, analyzing anaphylactic anti-OVA IgG1 Ab production measured by passive cutaneous anaphylaxis reaction, we detected that CpG failed to inhibit anaphylactic anti-OVA IgG1 Ab production following neonatal immunization, differently to adult immunized mice (Fig. 1b). CpG was also unable to modulate anti-OVA IgG1 Ab production, detected by ELISA, in both ages groups (Fig. 1c). In contrast, CpG induced an enhancement in the anti-OVA IgG2a Ab levels in immunized neonates, at similar levels than adult mice treated with CpG (Fig. 1d). These findings showed that CpG-ODN is able to downmodulate the development of IgE response in neonatal and adult phases, whereas its effectiveness was less pronounced in the neonatal allergen sensitization.

### CpG-ODN Induces Th1 Response in Neonatal Immunization with OVA

Although CpG-ODN administration in adult mice is known to induce Th1 cytokines, such as IL-12 and IFN-γ [24], it is unclear how CpG modifies the cytokine pattern on Th2-skewed immune response of allergen-sensitized neonates. We next assessed in vitro cytokine production upon various stimuli by spleen cells from OVA-immunized mice at neonatal period (Fig. 2). A marked increase in the IFN-γ secretion induced by anti-CD3 (Fig. 2a) stimulus was observed in mice immunized with OVA and CpG when compared to untreated immunized mice. However, there was no difference when the spleen cells were cultured with OVA. No difference was detected either in the IL-4 (Fig. 2b) or IL-10 (Fig. 2c) secretion levels between mice groups. These results show that downregulation of IgE response induced by CpG in neonatal period was concomitant to Th1 cytokine induction, whereas this adjuvant does not affect Th2-cytokine secretion.

CpG-ODN Suppresses IgE Development in the Neonatal Sensitization with the Dust Mite *Blomia tropicalis*

To assess the CpG-ODN effect on the development of IgE response to the dust mite Bt, mice were immunized with Bt extract and CpG at neonatal and adult ages. In the neonatal immunization, differently to OVA, Bt failed to induce detectable IgE Ab with anaphylactic activity, and for this reason total IgE Ab levels were shown. Figure 3a, b shows that CpG-ODN was able to downmodulate total IgE Ab levels in neonate and adult immunized mice, as well as anti-Bt IgE Ab production in adult immunized mice. In addition, these mice showed decreased anti-Bt IgG1 Ab levels (Fig. 3c). As expected, CpG-ODN underwent isotype switching to IgG2a Ab production in both neonate and adult immunized mice groups (Fig. 3d).

Fig. 1 CpG-ODN reduces IgE production in neonatal and adult immunization with OVA. Newborn (3 days old) and adult (6 weeks old) A/Sn mice were immunized with OVA, OVA plus CpG-ODN, or control-ODN, as described in the methods. The titers of anti-OVA anaphylactic IgE antibody were determined by individual analysis of serum samples by PCA (**a**). Anti-OVA anaphylactic IgG1 antibody was evaluated by analysis in triplicate of serum pool (five animals) by PCA reaction (**b**). The titers of anti-OVA IgG1 (**c**) and IgG2a (**d**) antibodies were determined by ELISA. The *dashed line* represents the detection limit of the assay. The results represent the mean±SEM of six to eight animals per group. *$p \leq 0.05$ compared to OVA group
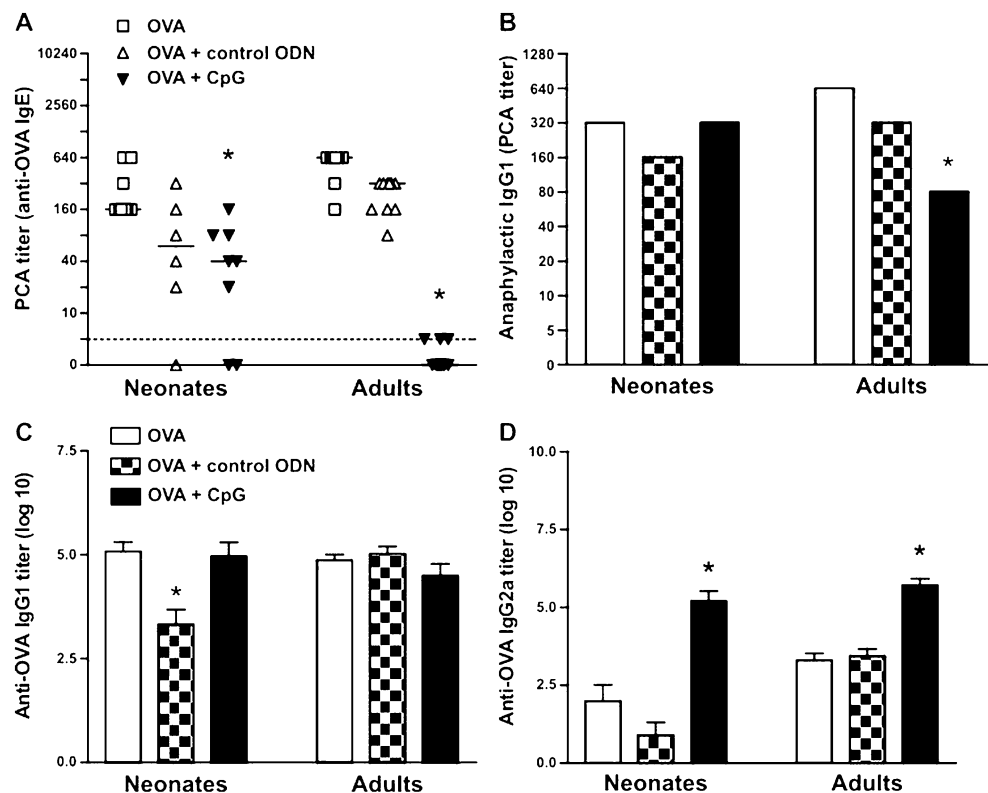
Although there is a differential allergenic potency between OVA and Bt, CpG was able to prevent the IgE development in both murine immunization models. Next, we evaluated whether CpG-ODN was effective to modify IgE response, when adult mice received coimmunization with OVA and Bt extract. Mice immunized with both Bt and OVA developed higher anti-OVA IgE Ab levels with anaphylactic function than with Bt alone, revealing an OVA-immunodominance over Bt (Fig. 3e). Nevertheless,

CpG was able to simultaneously inhibit IgE Ab production to both OVA and Bt allergens. Again, CpG-ODN did not interfere in either the anti-OVA or anti-Bt IgG1 Ab levels (Fig. 3e) while promoted an enhancement of IgG2a Ab response (Fig. 3f) in mice coimmunized with OVA and Bt. These results indicate the immunomodulatory potential of CpG-ODN on the type I hypersensitivity response in allergen-monosensitized or allergen-polysensitized mice.
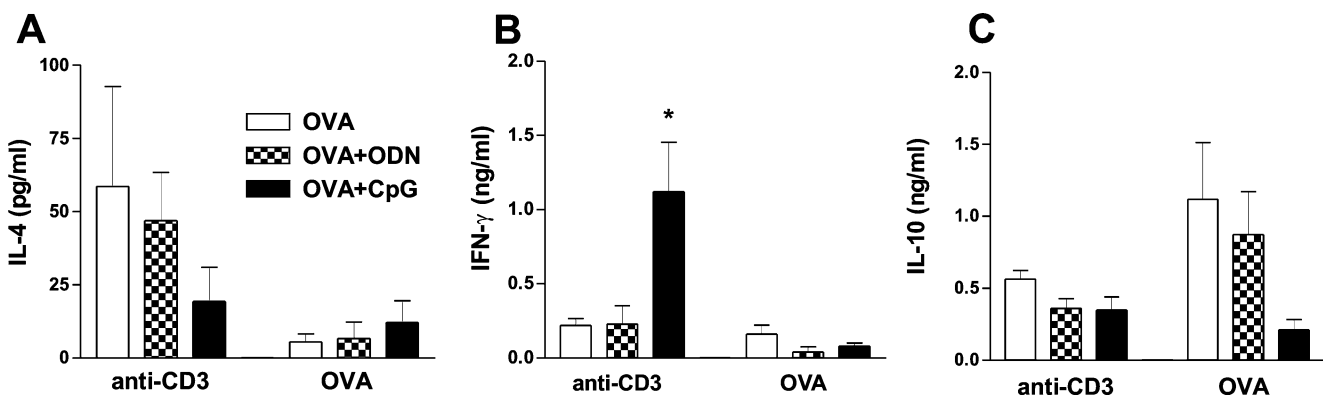

Fig. 2 CpG-ODN in neonatal immunization with OVA promotes IFN-γ production upon polyclonal stimulation in vitro. Three-day-old mice were primed with OVA and boosted 10 days later. Groups of mice received either CpG-ODN (OVA + CpG) or control-ODN (OVA + ODN) in both priming and boost with OVA. After 10 days, the mice were sacrificed. Splenocytes were cultured for 18 or 72 h with anti-CD3 mAb (1 μg/ml) or OVA (200 μg/ml), respectively. Supernatants were harvested for IL-4 (**a**), IFN-γ (**b**), and IL-10 (**c**) measurement by ELISA ($n=5$–9 per group). The *bars* represent the mean ± SEM of cytokine production after stimuli minus basal level. *$p \leq 0.05$ compared to OVA group

Fig. 3 CpG-ODN decreases IgE Ab production in Bt immunization and coimmunization with Bt and OVA. **a–d** Neonate (3 days old) and adult (6 weeks old) A/Sn mice were immunized with either Bt or Bt plus to CpG-ODN or control-ODN, as described in the methods. Total IgE concentration was determined by individual analysis of serum samples by ELISA (**a**), and the titers of anti-Bt IgE were determined by PCA (**b**). Titers of anti-Bt IgG1 (**c**) and IgG2a (**d**) antibodies were determined by ELISA. **e–f** Adult mice were coimmunized with OVA and Bt and boosted with both antigens 14 and 21 days after priming. Groups of mice received either CpG-ODN (OVA/Bt + CpG) or control-ODN (OVA/Bt + ODN) in the priming with OVA and Bt. The *dashed line* represents the detection limit of the assay. The *bars* represent the mean ± SEM of six to eight animals per group. *$p \leq 0.05$ compared to OVA group
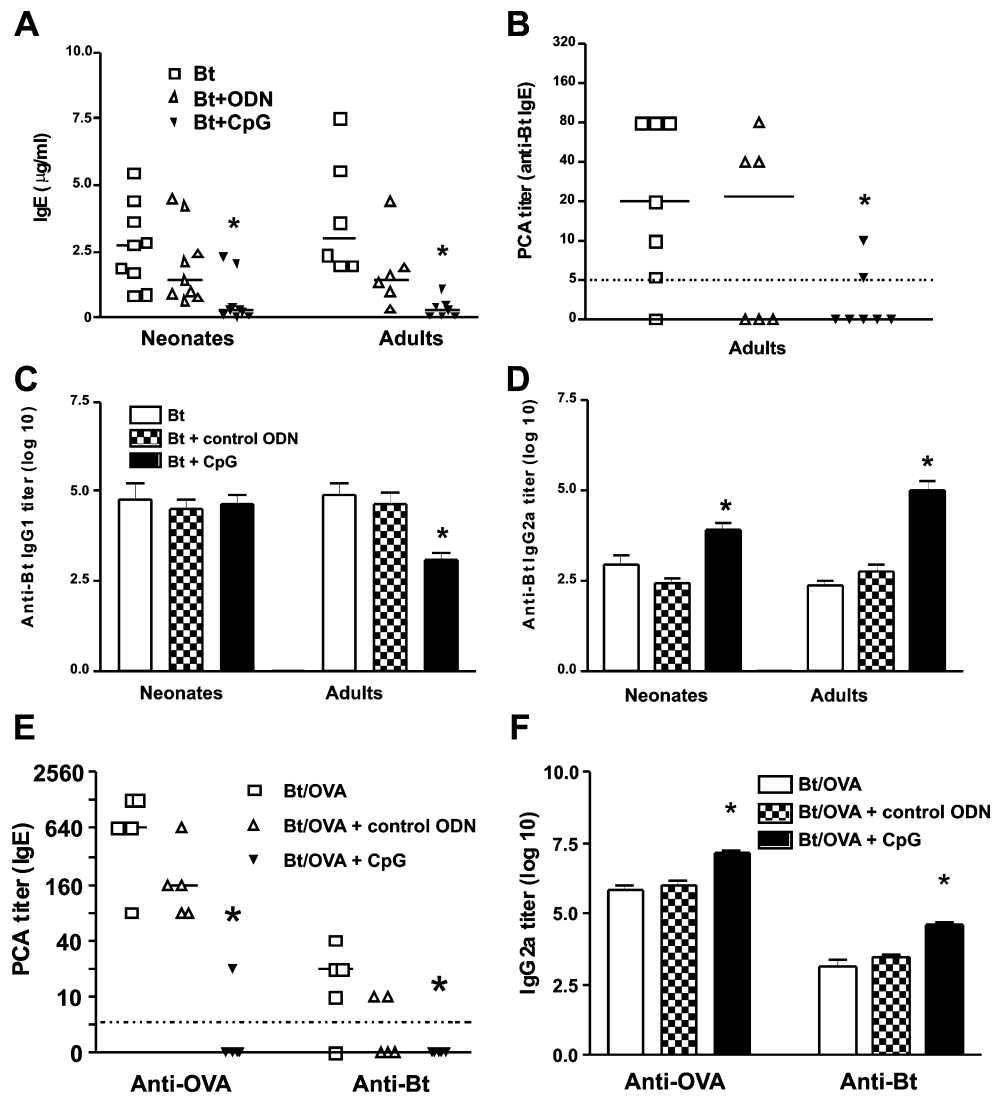
Altered TLR-9 Expression on B Cells from Neonate Mice After In Vitro CpG Stimulation

As observed, CpG-ODN in the neonatal allergen sensitization does not display fully immunomodulatory function on the Ab production when compared to adult mice. To verify whether the lower CpG-ODN efficacy to modulate the Ab response in the neonatal immunization could be related to the CpG receptor expression on B cells, we assessed TLR-9 expression by flow cytometry on splenic B cells from 10-day-old and adult nonimmunized A/Sn mice.

TLR-9 expression on B cells was undetectable in nonstimulated B cells, either from neonate or adult mice. In fact, resting B cells have very low TLR-9 expression, which augments the cellular activation that follows [28, 29]. Figure 4 shows that upregulation of TLR-9 expression from adult mice occurs in approximately 20% of B cells at 48 h after CpG stimulation, while B cells from 10-day-old mice did not change their TLR-9 expression, found in up to

1% of B cells. This B cell percentage remained low at least until 72 h after CpG stimulation (data not shown).

We also examined the effect of CpG-ODN on the *Tlr9*, *Ifn-γ*, and *Il10* mRNA expression in splenic B cells by real-time PCR. Figure 4b, c shows B cell mRNA expression after 3 h of CpG stimulation, where adult mice showed a peak of *Ifn-γ* mRNA. This effect was not observed on the neonatal B cells, which expressed increased levels of *Il10* mRNA. No difference in cytokine expression of B cells was detected either in 30 or 90 min of CpG stimulation. These findings showed that neonatal B cells were less sensitive to *Tlr9* signaling effects compared to adult B cells.

In order to verify if the lack of TLR-9 upregulation in neonate B cells could implicate in low functional responses, we analyzed the expression of activation markers B7.1 and B7.2 and cytokine production. In the culture of spleen cells stimulated with CpG (Fig. 5a, b), we observed decreased expression of B7.2 in B cells from neonate mice compared to cells from adults. However, when we stimulated purified
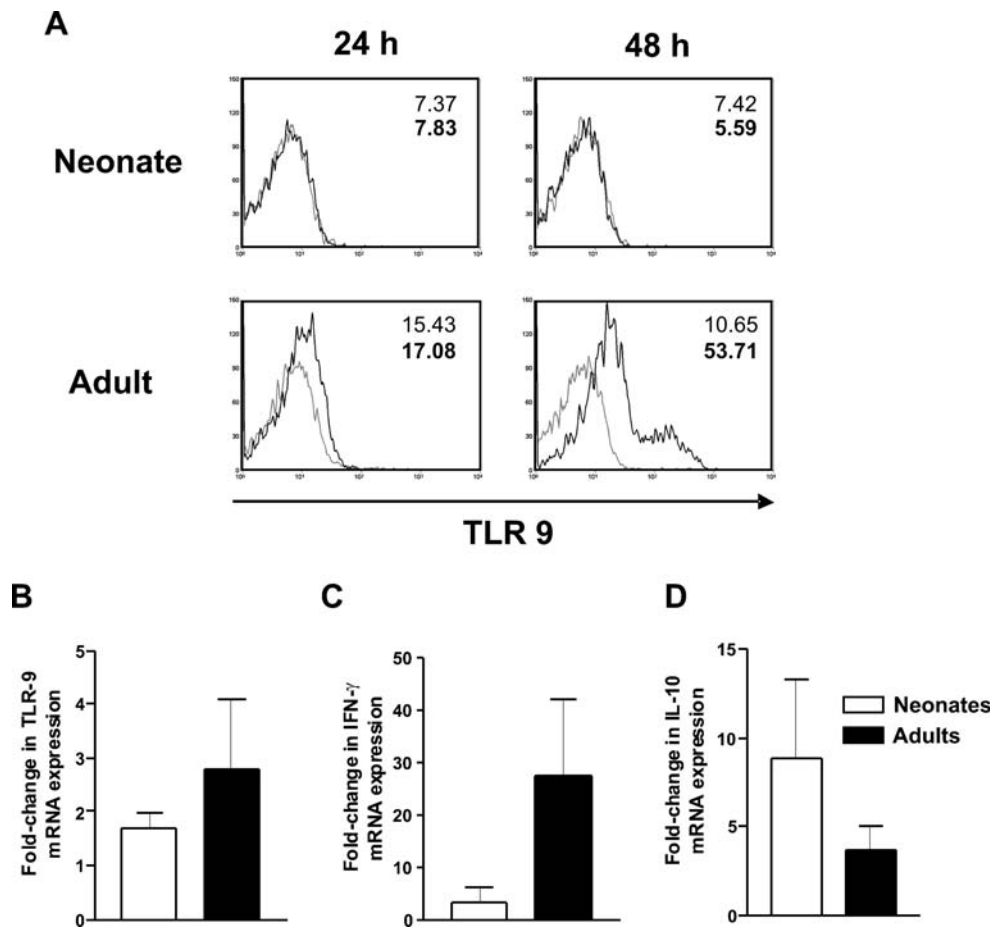
Fig. 4 Altered TLR-9 and IFN-γ expression on neonatal B cells upon CpG stimulation. **a** Splenocytes of 10-day-old and adult A/Sn mice (two experiments, pool of two animals/experiment) were cultured with CpG-ODN. The figure shows a histogram from one experiment of intracellular expression of TLR-9 in B220+ population (10,000 events) evaluated by flow cytometry after 24 and 48 h of CpG stimuli. The *numbers* in the histogram represent MFI values of nonstimulated (*upper*) and stimulated (*lower*) cells. **b** Purified splenic

B cells of 20-day-old (three experiments, $n=3$ animals per experiment) or 6-8-week (three experiments, $n=3$ animals per experiment) A/Sn mice were cultured 3 h with CpG-ODN stimuli (10 µg/ml). The analysis of TLR9, IFN-γ, and IL-10 mRNA expression and β-actin control was done by real-time PCR with SYBR Green. The results are expressed as the mean ± SEM of fold change in expression of cytokine in B cells stimulated with CpG compared to nonstimulated cells

B cells (Fig. 5c, d), the expression of B7.2 in adults was similar to neonates. Interestingly, the expression of B7.1 and B7.2 molecules was lower in purified B cells than in total spleen cells both in neonates and adults, suggesting that other cell types may contribute to the activation of B cells, probably by cytokines induced by CpG. Concerning the cytokine production by CpG-incubated purified B cells, an opposing effect was observed, with decreased IL-6 (Fig. 5e) and increased IL-10 (Fig. 5f) in neonates.

Upregulated B7 Molecule Expression on T Cells from Neonate Mice upon Antigen Stimulation Is Inhibited by CpG-ODN

The purpose of these experiments was to evaluate whether CpG may act indirectly on T cells during in vitro Ag-specific stimulation. B7 molecule expression on T cells has

been related to play an inhibitory role in neighboring cells [30, 31]. We examined the kinetic of T cell costimulatory molecule expression following OVA and CpG-ODN stimulation in spleen cell cultures of 10-day-old and adult DO11.10 OVA TCR-Tg mice.

Interestingly, an upregulation of B7.1 and B7.2 molecule expression on CD4+ T cells from neonate mice 72 h after OVA stimulation was observed when compared to adults (Fig. 6a, b). In neonate mice, B7 expression in Ag-stimulated T cells was approximately 15 times greater than in nonstimulated cells whereas B7 expression in adults was kept similar to the baseline. In addition, CpG-ODN was able to significantly decrease both B7.1 and B7.2 molecule expression on TCD4+ cells from neonate mice.

CD4+ T cells from neonates and adults expressed ICOS molecules at similar levels upon OVA stimulation, but that

Splenocytes

Purified B cells
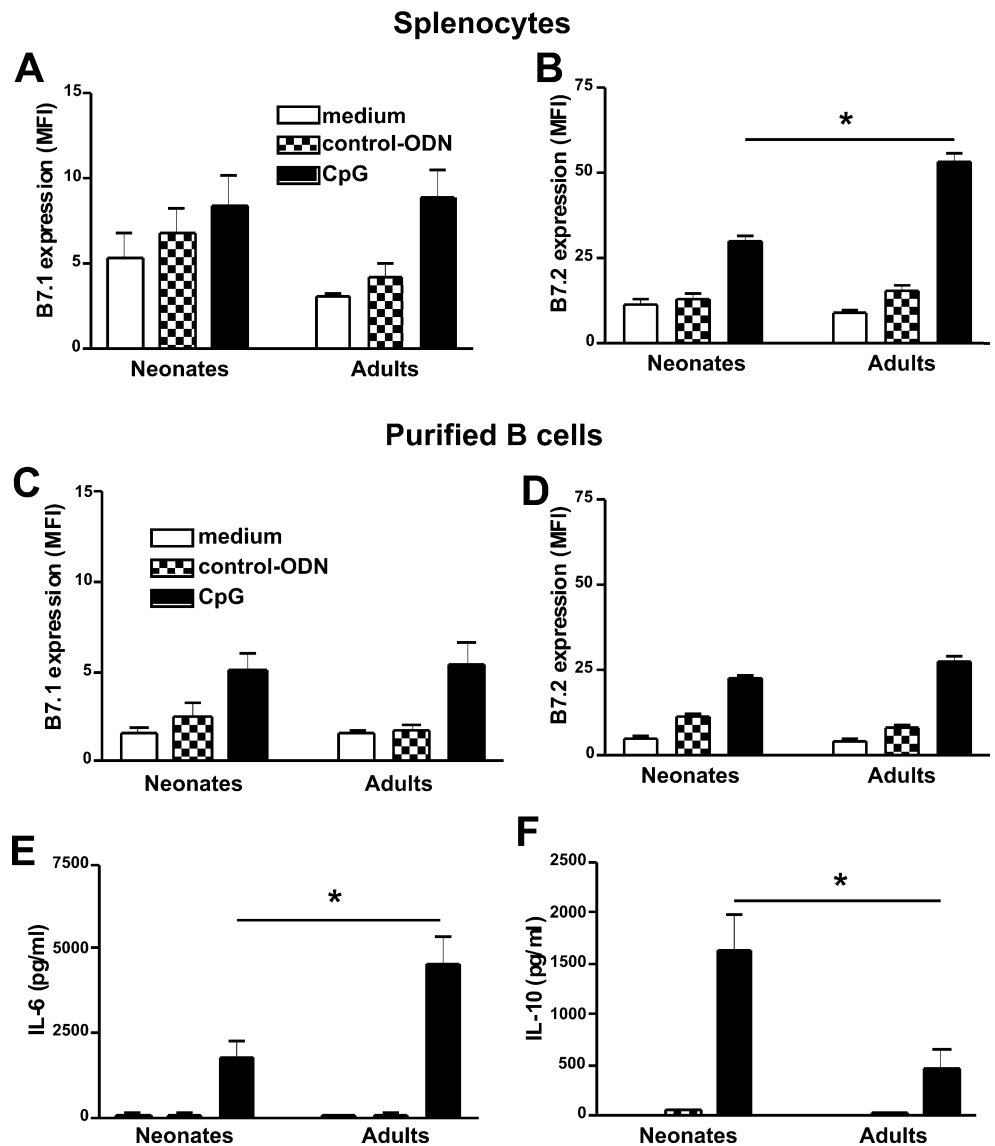
was markedly reduced in the presence of CpG (Fig. 6c). However, negative regulation of ICOS expression on CD4 cells from adult mice occurred at the lowest CpG concentration tested (0.1 μg/ml), while tenfold higher concentration of CpG was necessary to downmodulate ICOS expression on T cells from neonate mice.

Next, we determined the cytokine production upon in vitro OVA stimulation in the presence of CpG by spleen cells from neonate and adult Tg mice. Figure 7a shows that neonates produced less amounts of IFN-γ− induced by OVA than adults, whereas CpG significantly enhanced its secretion in both age groups (Fig. 7a). Neonates have a delayed IL-4 secretion compared to adults, reaching a peak 72 h after Ag stimulation, in similar amounts detected at 48 h by adult spleen cells (Fig. 7b). Inhibition of IL-4 secretion by CpG has been detected even at the lowest concentration (0.1 μg/ml) in both mice groups. Scarce IL-10 secretion levels were induced by Ag stimulation in both

neonate and adult spleen cells, which were intensively enhanced by CpG, irrespective of the dose employed (Fig. 7c). These findings showed a differential B7 expression on neonatal T cells that could be strategically reversed by the CpG-ODN.

Discussion

In this work, we show the immunomodulatory effect of CpG on the development of type I hypersensitive response to allergens inducing Th1-type response in both neonatal and adult immunization. The lower efficacy of CpG modulation in neonatal allergen sensitization suggests that some aspects of the newborn immune system, such as altered expression of TLR-9 by B cells, may affect innate responses, especially to PAMPs such as CpG. Interestingly, we also showed there is a higher expression of antigen-
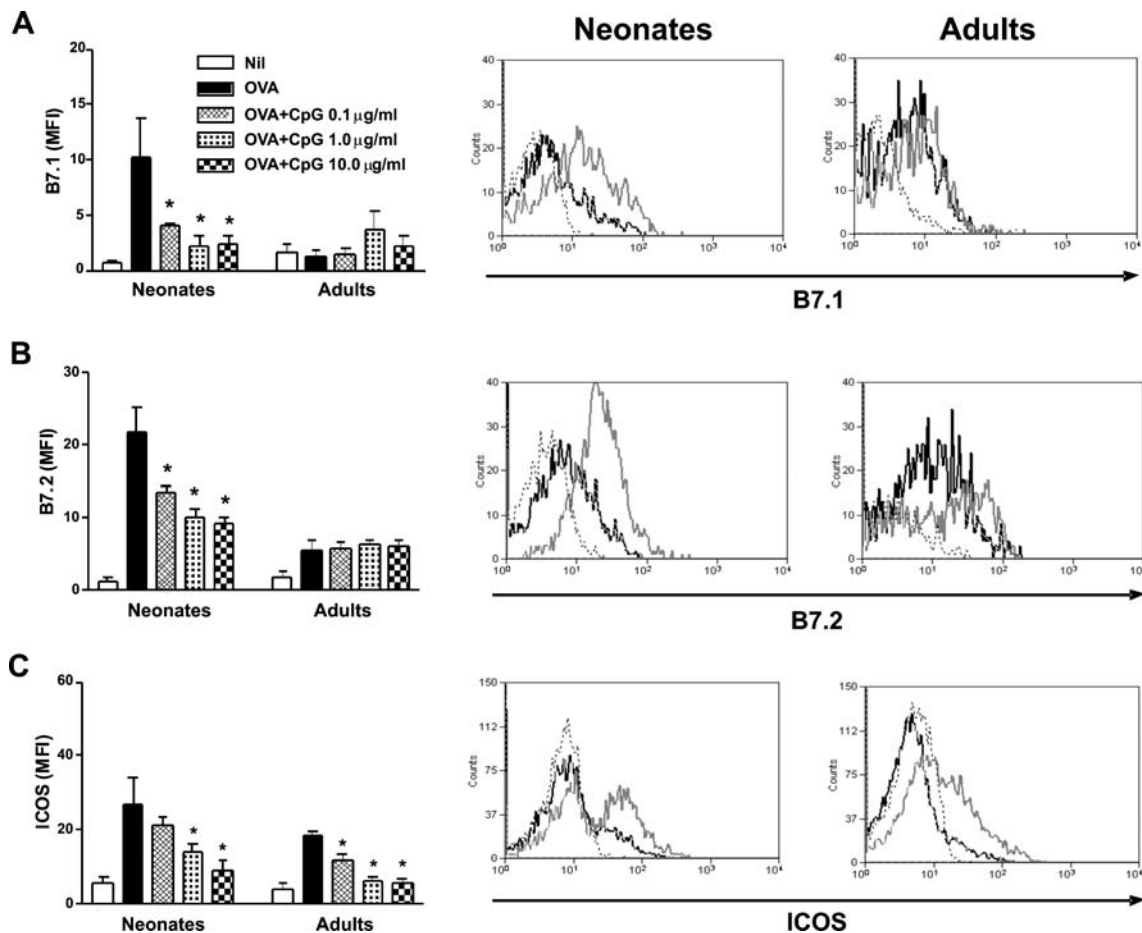
Fig. 6 Upregulation of B7 expression on CD4+ T cells of neonate mice is suppressed by CpG-ODN. Splenocytes from either 10-day-old (three to seven experiments, $n=3$ animals per experiment) or 6–8-week-old (six to ten animals, three to five experiments) DO11.10 transgenic mice were cultured for 72 h with OVA (100 μg/ml) in the presence of different concentrations of CpG-ODN (0.1, 1.0, and 10 μg/ml). Mean fluorescence intensity (*MFI*) expression of B7.1, B7.2, and ICOS molecules in CD4+ T cells was evaluated after 24, 48, and 72 h, by flow cytometry, in CD4+ population (10,000 events). The *bars* represent the mean ± SEM. *$p \leq 0.05$ compared to OVA stimuli. The *right panels* illustrate the analysis of B7 and ICOS expression in CD4+ T cells simulated with either OVA (*gray line*) or OVA + CpG-ODN (*dark line*) for 72 h. The *dashed line* represents nonstimulated cells

induced B7 molecules on newborn CD4+ T cells than in adult counterparts and that CpG was able to reverse this enhanced B7 molecules expression.

In mice, the newborn period is marked by a skewed Th2 response that contributes to allergic response development [32]. Thus, maturation of Th1-related responses in early life becomes a strategy to avoid Th2 allergen priming and consequently allergy development. We observed that administration of CpG-ODN on sensitization with OVA or Bt decreased IgE production, a Th2-dependent isotype. Among OVA-immunized mice that received CpG, the levels of IgE were higher in newborns in comparison to adults. Moreover, anaphylactic IgG1 titers were suppressed only in adult mice. Newborn mice that received CpG were not able to modulate IL-4 as well anaphylactic IgG1 Ab synthesis, possibly because anaphylactic IgG1 is IL-4 dependent [18]. The low IgE and high IgG2a levels in

newborn immunization may be, at least in part, due to an enhanced IFN-γ production induced by CpG. This effect is also observed in other models of newborn vaccination, where BALB/c mice immunized with hepatitis B surface antigen associated to CpG-ODN showed increased IgG2a and IFN-γ production followed by a decrease of IgG1 Ab levels [33].

The disparity in responses of newborns and adults is likely to occur due to a lower efficiency of CpG modulation in early life. Probably, for the maintenance of inhibited IgE response, further CpG injections could be needed. In agreement with CpG downmodulatory effect on allergen sensitization, CpG was also able to simultaneously inhibit IgE levels in mice immunized with two allergens (i.e., OVA and *B. tropicalis*). OVA is a dietary antigen known to induce strong immune response with a Th1/Th2 mixed pattern; Bt is a mite that may cause sensitization in atopic
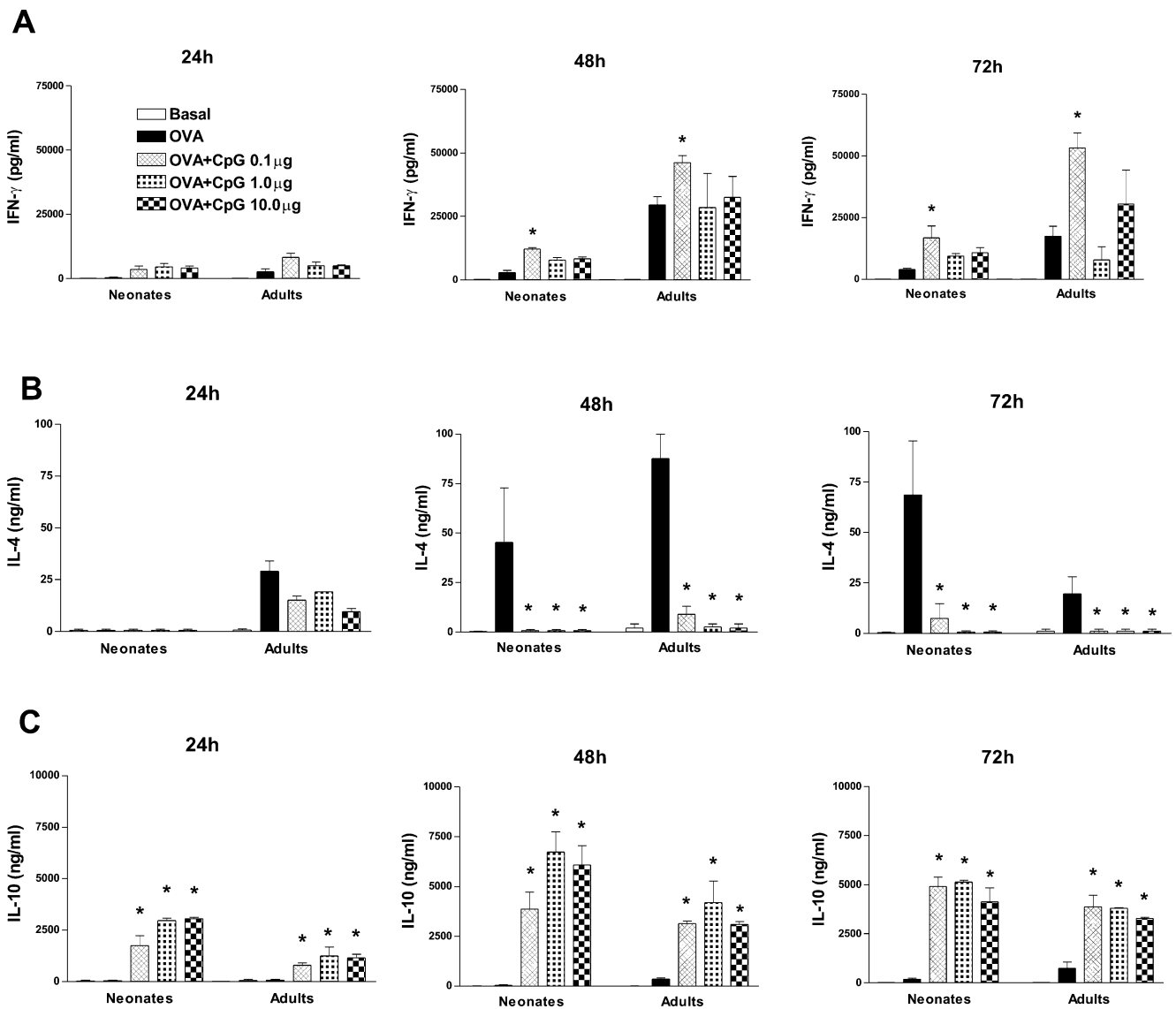
Fig. 7 CpG-ODN inhibits IL-4 secretion by induction of IFN-γ. Splenocytes of either 10-day-old (four experiments, $n=3$ animals per experiment) or 6–8-week-old (three experiments, $n=2$ animals/experiment) DO11.10 mice were cultured for 72 h with OVA (100 μg/ml) in the presence of different concentrations of CpG-ODN (0.1–10 μg/ml). After 24, 48, and 72 h, supernatants were collected, and concentrations of IFN-γ (**a**), IL-4 (**b**), and IL-10 (**c**) were determined by ELISA. The *bars* represent the mean ± SEM. *$p ≤ 0.05$ compared to OVA stimuli

individuals [34] and promotes IgE production and lung inflammation with neutrophil infiltration in A/Sn mice [35, 36]. Moreover, Bt presents allergen peculiarities such as the presence of fractions that can induce T-independent responses [35], which can explain the lack of primary IgE response on newborn immunization. Together, this emphasizes CpG effectiveness on the allergen polysensitization, which is clinically relevant since a high prevalence of monosensitization as well as polysensitization can be found in atopic children [37].

In neonates immunized with OVA, scarce amounts of antigen-induced cytokines were found; thus, we verified the effect of CpG using spleen cells from DO11.10 transgenic mice. In fact, CpG induces IFN-γ secretion, mainly in adults, whereas efficiently inhibited IL-4 production by both neonate and adult Tg mice. It addresses an interesting approach to the predisposition of the Th2 response in the neonatal period. Interestingly, CpG induced an early increase of IL-10 secretion. We speculate that the source of enhanced amounts of Ag-induced IL-10 should be both T and B cells. In fact, an increased IL-10 mRNA expression was detected on purified B cells of newborn mice. In the neonatal period, there are a large number of CD5+ B-1 cells that are able to produce high amounts of IL-10 after

activation of TLR-9 by CpG [38]. Indeed, it may affect Th1 response development, as in the experimental alloimmune response, where dendritic cell functions are suppressed [39].

The modulation of CpG on antibody production may be also mediated in a Th1-cytokine-independent manner, possibly by direct activation of effector B cells. It has been described that CpG recognition by B cells through TLR-9 induces t-bet expression and regulates immunoglobulin class switching, decreasing IgG1 and IgE while increasing IgG2a Ab production [16]. Moreover, it was demonstrated that B cells from TLR-9- or MyD88-deficient mice are not able to produce, in vitro, isotypes dependent of Th1 cytokines after CpG stimulation. In our work, the type of CpG-ODN (ODN 1826) used activates mainly B cells [15], exerting an important role in increasing antigen-specific IgG2a Ab levels in immunized mice.

We verified that, in early life, B cells are less activated and produces lower amounts of IL-6 upon CpG stimulation in comparison to adult mice. This fact could be associated to the impaired TLR-9 upregulation, contrary to adult mice. These results suggest that CpG might be less effective in early life than in adulthood due to a defective expression of TLR-9 after agonistic stimuli, which causes a lower sensitivity of B cells. Up to now, there are no data about this defective expression of TLR-9 in young mice compared to adult. Further, we detect increased IFN-γ mRNA expression only in adult B cells. It has been suggested that IFN-γ production by B cells contributes towards Th1 response differentiating in a similar way to CD4+ T cells, based on the cytokine pattern [40, 41]. In response to IL-12, B cells produce IFN-γ, which induces the expression of t-bet and consequently the increase of IL-12R [42]. The inability of neonatal B cells to produce IFN-γ may result in a reduction in IL-12 production or in expression of α and β IL-12R subunits, which are necessary for IL-12 signaling.

Interestingly, neonates showed a pronounced increase in expression of B7.1 and B7.2 molecules in CD4+ T cells upon stimulation, which may be a factor favoring low immune response and high predisposition to tolerance in the neonatal period. Some studies related the interaction of B7.1/B7.2 of CTLA-4 in a T cell–T cell fashion as an important control mechanism of activated T cell proliferation and survival by inhibiting alloreactive response [30] and T cell stimulation with high concentrations of concanavalin A [31]. However, until now, differences in B7 expression between neonatal and adult murine T cells following Ag-specific stimulation have not been described. Furthermore, CpG reverses upregulation of B7.1/B7.2 expression as well as ICOS induced by Ag on CD4+ T cells. It is possible that a decrease of B7 and ICOS molecules by CpG could be involved in the survival of CD4+ T cells of both neonate and adult mice since it has

been claimed that activated CD4+ T cells express TLR-9 mRNA and that CpG directly promotes T cell survival [42]. In our data, it seems that a suppression of ICOS expression by CpG in both neonates and adults may play a role in the Th1 directing response. It has been described that, in vitro, ICOS expression is inhibited by IL-12, and, further, some works suggests that ICOS plays an important role in the Th2 response and T regulatory cells [43].

Our results showed the immunomodulatory potential of CpG in newborns and in adults of the IgE response to OVA and Bt. We also demonstrated qualitative and quantitative effects with regards to CpG modulation of neonatal and adult immunization. Newborn susceptibility to infections and allergy development points to the importance of understanding the neonatal immune system to establish immunomodulatory strategies potentiating innate and adaptive responses for prevention of allergic diseases.

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