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Original Article

Dendritic cells expressing MyD88 molecule are necessary and sufficient for CpG-mediated inhibition of IgE production *in vivo*

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Abstract: Elevated levels of immunoglobulin E (IgE) are associated with allergies and other immunological disorders. Experimentally, sensitization with alum adjuvant favors IgE production while CpG-ODN adjuvant, a synthetic toll-like receptor 9 (TLR9) agonist, inhibits it. The cellular mechanisms underlying TLR-regulation of immunoglobulin production are still controversial. Specifically, TLR-mediated IgE regulation *in vivo* is not yet known. We show that augmented levels of IgE induced by sensitizations to OVA with or without alum adjuvant or with OVA-pulsed dendritic cells (DCs) were inhibited when sensitization to OVA was performed in the presence of CpG. Notably, CpG-mediated suppression of IgE production required *MyD88*-expression on DCs but not on B-cells. This contrasts with previous reports of *in vitro* regulation IgE where CpG acted directly on B cells via MyD88 pathway. In addition, CpG also inhibited IgE production in a *MyD88*-dependent manner when sensitization was performed with OVA-pulsed DCs. Finally, CpG signaling through MyD88 pathway was also necessary and sufficient to prevent anaphylactic antibody production involved in active cutaneous anaphylaxis.

Keywords: Allergy, IgE, IgG2c, anaphylaxis, dendritic cells

1. Introduction

Over the past five decades the incidence and prevalence of allergic disease worldwide have been increasing. Atopy is a term used to describe a group of diseases such as allergic rhinitis, hay fever, asthma, atopic eczema and food allergy in individuals that are genetically predisposed to develop an immediate immunoglobulin E (IgE)-mediated hypersensitivity to otherwise harmless environmental antigens [1]. The work of Shirakawa et al. showing that among Japanese schoolchildren, there was a strong inverse association between serum levels of IgE and delayed type hypersensitivity to Mycobacterium tuberculosis antigens [2] lend support to the "hygiene hypothesis" that postulated an inverse relationship between allergy and infections [3]. Classically, IgE and IgG1 antibody switching is mediated by interleukin-4 (IL-4)-producing T helper 2 (Th2) cells while interferon gamma (IFNγ)-producing Th1 cells favor IgG2a switching [4]. Therefore, it is expected that Toll-like receptors (TLRs) agonists that are viewed as Th1 adjuvants would induce Th1-associated isotypes while alum, that is considered a Th2 adjuvant, would favor IgE production [5]. However, despite its Th2-promoting activities, alum has been used empirically in many anti-microbial vaccines formulations [6]. We have previously shown that adsorption of lipopolysaccharide (LPS), a TLR4 agonist, to alum-based tetanus toxoid vaccine dampens toxoid-induced IgE production but enhances IgG1 and IgG2a antibody production [7]. More recently, using a similar approach of absorbing TLRs agonists to alum, we found the TLR9 agonist composed of oligodeoxynucleotides (ODN) containing CpG motifs (hereafter referred to as CpG) was the most effective among the TLRs agonists in dampening IgE production [8]. Since CpG is another TLR adjuvant approved for use in humans [9] we were interested in dissecting the mechanism(s) by which alum-based CpG formulation regulates immunoglobulin class switching. Moreover, studies to ascertain the molecular mechanisms governing IgE production and its regulation by adjuvants *in vivo* have not been addressed. Here we used an

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established OVA-model of respiratory allergy that course with high levels of total and OVA-specific IgE and low levels of IgG2c antibodies and focused on the role of MyD88 molecule expressed on CD19-positive B cells or on CD11c-positive DCs in CpG-mediated regulation of isotype class switching. We show that addition of CpG to OVA/alum suppressed IgE production and increased IgG2c production in mice selectively deleted for MyD88 molecule in B cells but not in DCs indicating that MyD88-expressing DCs are necessary and sufficient to suppress IgE and enhance IgG2c production induced by sensitization with CpG in the formulation.

2. Materials and Methods

Animals

Six-to-eight-week-old female C57BL/6 mice (WT), MyD88-KO, RAG-KO or Myd88 fl/fl and mice expressing the recombinase Cre under the control of the CD19 or Itgax (CD11c) promoter [10] were originally purchased from Jackson Laboratories (Bar Harbor, ME) and kept at a specific pathogen-free breeding unit at the Institute of Biomedical Sciences of the University of Sao Paulo (ICB IV-USP). CD11cMyD88$^{-/-}$ (DC-MyD88$^{-/-}$) or CD19cMyD88$^{-/-}$ (B-MyD88$^{-/-}$) mice with specific deletion of MyD88 adaptor molecule in CD11c-positive or CD19-positive cells were generated by breeding respectively CD11c-Cre or CD19-Cre with Myd88flox mice. Proper littermates for the DC-MyD88$^{-/-}$ group (CD11cMyD88$^{+/+}$, hereafter referred as DC-MyD88$^{+/+}$) were also generated. All mice (five animals per cage with a ventilated system Alesco, Monte Mor, São Paulo, Brasil) were kept in 12h light/dark cycle, temperature controlled rooms, food and water ad libitum, and towel paper used for environment enrichment. Mice were treated according to animal welfare guidelines of ICB (Ethic Protocol 009/2015) under National Legislation-11.794 Law.

Alum gel preparation

The aluminum gel ($Al(OH)_3$) was prepared by precipitation of ammonium aluminum sulfate dodecahydrate ($AlH_4(SO_4)_2$-12 H_2O, Alfa Aesar, MA, USA) with an excess of 1N NaOH (Synth, SP, Brazil). Aluminum hydroxide (Alum) was suspended in water (Milli Q, Ontario, Canada), washed five times and centrifuged at 3000 rpm for 15 min. The final precipitate was suspended again in water and the final concentration was determined by calculating 1 mL of dry solution.

Experimental Protocol

Mice were sensitized subcutaneously (s.c.) with 4 μg of ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO, USA) or with OVA plus 10 μg CpG (CpG-ODN 2395 Class C, a TLR9 agonist purchased from Invivogen San Diego, CA, USA) with or without alum adjuvant gel (1.6 mg) on days 0 and 7. Mice were intra-nasally (i.n.) challenged with 10 μg of OVA in 40 μL of PBS on days 14 and 21. Control mice consisted of naïve non-manipulated animals. In some experiments mice were sensitized with bone marrow-derived dendritic cells pulsed with OVA or OVA plus CpG on days 0 and 7 (see methods bellow). All treatment procedures were done under anesthesia with Ketamine 10mg/kg (Rhobifarma, São Paulo, Brazil) plus Xylazine 100mg/kg (Rhobifarma, São Paulo, Brazil) in the intraperitoneal (i.p.) route diluted in PBS. Animals were euthanized by inhaled isoflurane (Cristália, São Paulo, Brasil) 24 h after the last challenge and samples were collected. The OVA used throughout the study was diluted in PBS (2 mg/mL) and depleted of the endotoxin (LPS) activity (measured by Limulus amoebocyte lysate QCL-1000 kit from BioWhittaker, Walkersville, MD, USA) using six to eight cycles of Triton X-114 extractions. The endotoxin level of purified OVA (2 mg/mL) was below the limit of detection of Limulus assay lysate (less than 0.1 Endotoxin Units).

Generation of Bone Marrow Derived Dendritic Cells

Briefly, bone marrow-derived dendritic cells (BM-DCs) were obtained from femurs of C57BL/6 or DC-MyD88$^{-/-}$ mice. Both ends of the femurs were cut after removing the femurs under sterile

conditions and femur was flushed with a syringe containing 5 mL of RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA). Bone marrow cells were plated into 6-well plates at a concentration of 1×10^6 cell/well and cultured at 37ºC in 5% CO_2 from day 0 to 4 with 20 ng/mL of GM-CSF (BioLegend, San Diego, CA, USA) and supplemented with more GM-CSF on day 4 [11]. On day 6, the cells were primed with OVA (100ug/well) or OVA plus CpG (1ug/well). On day 7, BM-DCs were harvested, washed with PBS, counted and 1×10^6 cells were used for the subcutaneous sensitization.

ELISA For Antibody Determinations

Blood samples were collected by cardiac puncture, centrifuged and serum was stored at −20°C. Serum antibodies were determined by Enzyme-linked immunosorbent assay (ELISA). Total IgE concentrations were determined by sandwich-ELISA using kit BD OptEIA ELISA Set (BD, San Diego, CA, USA). OVA-specific IgE levels were determined by adding serum samples at 1/10 dilutions to plates with rat anti-mouse IgE (SouthernBiotech, Birmingham AL, USA). After washing, biotin-labeled OVA was added and revealed with ExtrAvidin (Sigma-Aldrich, St. Louis, MO, USA) plus substrate. OVA-specific IgE serum concentrations were deduced using Chondrex kit (Chondrex, WA, EUA) with known concentrations of OVA-specific monoclonal IgE antibody. OVA-specific IgG1 and IgG2c were measured by coating the plates with 20 μg/mL of OVA. Serum samples were added at multiple dilutions and revealed with goat anti-mouse IgG1 or IgG2a conjugated to HRP (SouthernBiotech, Birmingham AL, USA), which also reacts with IgG2c isotype. Standard OVA-specific curves for IgG1 and IgG2c were purchased from Chondrex. All ELISA were performed in 96-well maxisorp plates (Thermo Fisher Scientific, Rochester, NY).

Active Cutaneous Anaphylaxis (ACA) Assay

Mice were sensitized as described above and challenged intranasally on day 14. On day 20, the dorsal region was shaved with a trimmer ER389 (Panasonic, Japan). Twenty- four hours later, the animals received an intradermal injection of OVA (10ug) or PBS and immediately after an intravenous injection of 100 l of Evans Blue dye (1mg/mL). Thirty minutes later, the animals were sacrificed and skin of the dorsal region was removed for photographic registration. Skin spots were excised, weighted and dye extraction performed with formamide (8ml/mg of dry weight) for 72 hours [12] and quantified by measuring dye absorbance at 620 nm. Results are expressed as g/mL determined by a standard curve with known concentrations of Evans blue dye.

Statistical Analysis

Statistical analyses were performed using GraphpadPrism (V.5; GraphPad Software, USA). One-way ANOVA followed by Tukey post-test was performed, as appropriate. Differences were considered statistically significant when p value ≤ 0.05. Data represent the mean ± SD.

3. Results

3.1. Sensitization to OVA in the presence of CpG inhibits IgE production and increases OVA-specific IgG2c isotype

To determine the influence of CpG on OVA sensitization and consequent OVA-induced airway eosinophilic allergic inflammation or OVA-specific antibody production we subcutaneously sensitized C57BL/6 mice to OVA or to OVA plus CpG in the presence or absence of alum adjuvant and then challenged with OVA as depicted in Fig. 1A. We found that sensitization to OVA induced eosinophilic allergic inflammation that was more intense in the presence of alum as revealed by total cell and eosinophil counts (Fig. 1B-C). Addition of CpG to OVA sensitization, in presence or absence of alum decreased allergic inflammation (Fig. 1B-C), confirming and extending previous results [8]. Analysis of antibody isotype production showed that sensitization to OVA with alum adjuvant

resulted in roughly 3-fold higher levels of total and OVA-specific IgE than that obtained in mice sensitized to OVA without alum adjuvant (Fig. 1D-E). In contrast, sensitization to OVA in the presence of CpG inhibited IgE production (Fig. 1D-E). Sensitization to OVA with alum induced respectively low and high concentrations OVA-specific IgG2c and IgG1 antibodies (Fig. 1F-G) while in mice sensitized to OVA without alum the concentrations of both OVA-specific IgG2c and IgG1isotypes were negligible (Fig. 1F-G). Notably, sensitization to OVA with alum in the presence of CpG significantly augmented OVA-specific IgG2c, but not IgG1 antibody production (Fig. 1F-G) while in mice sensitized to OVA without alum adjuvant, the production of OVA-specific IgG2c as well as OVA-specific IgG1 significantly increased compared to OVA group (Fig. 1F-G). Collectively, we conclude that high levels OVA-specific IgG1 production were obtained when alum adjuvant was used whereas in adjuvant-free conditions its production was only induced by addition of CpG. Therefore, it is clear that CpG negatively regulates IgE production while IgG2c production is positively regulated, independently of whether alum is used or not for OVA sensitization. Therefore, in all subsequent experiments we focused on IgE and IgG2c production.

3.2. MyD88 expression on B cells is not essential for CpG-mediated regulation of IgE and IgG2c isotype production *in vivo*

We first determined the requirement of MyD88 molecule using mice with complete lack of MyD88 molecule since CpG is thought to signal mainly via MyD88 pathway. We found that sensitization to OVA in the presence of CpG did not block IgE production in MyD88$^{-/-}$ mice when compared to WT mice (Fig. 2A-B), indicating the essential role of MyD88 molecule in mediating the inhibition of IgE production. In the same vein, CpG did not enhance IgG2c production in MyD88$^{-/-}$ mice (Fig. 2C). Therefore MyD88 molecule is essential for isotype class switching regulation by CpG. However, the target cell type involved in this regulation remained elusive. It has been shown *in vitro* that CpG acts directly on purified B cells inhibiting IgE production and enhancing IgG2a production [13]. Importantly, sensitization to flagellin (an antigen protein and a TLR5 agonist) adsorbed to alum, resembling our protocol (OVA+ CpG/alum) of sensitization, suggested that the generation of T cell-dependent antigen-specific antibody responses required the activation of B cells via MyD88 pathway [14]. Therefore, we next determined the effect of CpG in mice with selective deficiency of *Myd88* gene in B cells using Cre-lox technology to delete *Myd88* gene in CD19-positive cells (B-MyD88$^{-/-}$). We found that CpG inhibited IgE production in mice bearing *Myd88*-deficient B cells (Fig. 2A-B) clearly indicating that *Myd88* expression on B cells is dispensable for the inhibition of IgE production. We conclude that *Myd88* expression is fundamental for down regulation of IgE production by CpG, but *Myd88* expression on B cells is not necessary for CpG-induced IgE inhibition.

3.3. MyD88-expressing CD11c-positive dendritic cells molecule are necessary and sufficient for CpG-induced inhibition of IgE and augmented IgG2c production

Our results indicated that B cells were dispensable for CpG-mediated regulation of IgE and IgG2c isotypes. Therefore, we focused our study on the role of *Myd88* expression on other cell type that respond to CpG signaling and is involved antigen presentation, namely dendritic cells (DCs). For this we used mice with specific deletion of *Myd88* gene on cells expressing CD11c integrin (DC-MyD88$^{-/-}$). As control group we used WT and littermates mice with MyD88-expressing MyD88 CD11c-positive cells (DC-Myd88$^{+/+}$). Sensitization with OVA plus CpG adsorbed on alum inhibited IgE production in WT and DC-Myd88$^{+/+}$ littermates, but not in DC-Myd88$^{-/-}$ mice (Fig. 3A-B). Likewise, OVA-specific IgG2c production was increased in WT and DC-MyD88$^{+/+}$ but not in DC-MyD88$^{-/-}$ mice in OVA+CpG group when compared to OVA group (Fig. 3C). Therefore, we conclude that CpG signaling through MyD88 molecule expressed on CD11c^{+} putative DCs is necessary and sufficient for IgE inhibition and enhancement of IgG2c production *in vivo*. In order to characterize functionally anaphylactic antibody activity we determined the magnitude of active cutaneous anaphylaxis (ACA) that was induced in mice upon intradermal OVA administration in

WT or DC-MyD88$^{-/-}$ mice sensitized with OVA/alum or OVA+CpG/alum. We found that both WT and DC-MyD88$^{-/-}$ mice sensitized with OVA/alum developed ACA of similar magnitude as revealed by ACA score based on Evans blue dye extravasation (Fig. 3D, left panel and E). In contrast, sensitization with OVA+CpG/alum inhibited ACA in WT but not DC-MyD88$^{-/-}$ mice (Fig. 3, right panel and E). These results reinforce that CpG inhibits the production of anaphylactic antibodies.

3.4. CpG-induced inhibition of IgE and augmented IgG2a production is independent of type I or type II interferon receptors

We have previously shown that type I cytokines or type I and II interferon (IFN) receptors were not required for CpG-induced inhibition of IgE production [8], however we have not determined the regulation of IgG2a/c isotype in these experiments. Therefore we performed experiments using thee 129 mouse strain with deficiencies in type I or type II IFN receptors. The 129 strain mice carry the Igh^{a} haplotype and therefore produce IgG2a and not the IgG2c isotype [15]. We found that CpG inhibited IgE production in WT as well as in IFNAR$^{-/-}$ or IFNGR$^{-/-}$ 129 mice (Fig. 4A-B), confirming our previous results that type I or type II INF receptors are not involved in CpG-mediated inhibition of IgE [8]. Notably, we found that CpG augmented IgG2a antibody production in these mouse strains (Fig. 4C). We conclude that deficiency of type I or type II interferon receptors *per se* did not significantly influence the regulation of isotype class switching by CpG.

3.5. MyD88 expression on OVA-primed dendritic cells is essential for inhibition IgE production by CpG

Given the crucial role that MyD88 molecule expressed on DCs exerts on CpG-induced immunoglobulin class switching we next determined whether similar regulation could be obtained using purified DCs for sensitization. For this, bone marrow derived dendritic cells (BM-DCs) were differentiated *in vitro* from WT or DCs-*Myd88*$^{-/-}$ donor mice and were pulsed with OVA alone or OVA plus CpG and injected subcutaneously in WT or DC-*Myd88*$^{-/-}$ recipients respectively using the protocol depicted in Figure 5A. We found that sensitization with DCs pulsed with OVA, expressing or not MyD88 molecule, induced upon intranasal OVA challenge an increase of total and OVA-specific IgE antibodies upon intranasal OVA challenge (Fig. 5B-C). Notably, animals sensitized with WT BM-DCs pulsed with OVA plus CpG, but not with DC-Myd88$^{-/-}$, inhibited IgE production (Fig. 5B-C) indicating that *MyD88* expression on DCs is fundamental for CpG-induced IgE inhibition. Surprisingly, OVA-specific IgG1 antibodies were bellow detection in all groups groups studied (data now shown). Moreover, OVA plus CpG-pulsed DCs obtained from WT but not from DC-Myd88$^{-/-}$ donor mice induced very low concentrations (~5 μg/mL) of OVA-specific IgG2c antibodies (Fig. 5C). The results with OVA-pulsed DCs indicate that this type of sensitization is not sufficient to induce significant production of IgG isotypes and reinforce our results indicating that *Myd88* expression on DCs is necessary and sufficient for CpG-mediated inhibition of IgE production.

4. Discussion

In the present work we focused on the regulation of immunoglobulin class switching in mice sensitized to OVA in the presence or absence of alum or CpG. We found that independently of whether alum adjuvant was used for OVA sensitization, addition of CpG to OVA resulted in inhibition of total and OVA-specific IgE production and in augmented OVA-specific IgG2a/c production. Therefore, it is clear that CpG inhibits IgE and enhance IgG2a/c isotype production. The effect of CpG on OVA-specific IgG1 production was dependent on whether alum adjuvant was used or not for OVA sensitization. In alum-free OVA sensitization, OVA-specific IgG1 antibodies were almost absent while in sensitization to OVA in the presence of CpG, IgG1 levels were significantly higher than that observed with sensitization to OVA alone. In contrast, when alum was used for OVA sensitization, OVA-specific IgG1 production reached high levels that were not further increased significantly with the addition of CpG. Since OVA-specific IgG1 isotype was significantly

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increased in sensitization to OVA in the presence of CpG, a TLR9 agonist that is considered a pro-Th1 adjuvant in mice, the IgG1 isotype cannot be viewed solely as Th2-associated isotype [4]. In this regard, there is a controversy in the literature as to whether TLR signaling is fundamental or not for enhanced production of IgG isotypes [16,17]. Our results reconcile in part this controversy since sensitization to OVA with alum adjuvant induces high levels OVA-specific IgG1 antibodies and as such its production seems to be TLR-independent, a finding that is in line with the work of Gavin et al. [17] while in alum-free sensitization, substantial levels of OVA-specific IgG1 production only occurred in the presence of CpG indicating the involvement of TLR signaling in IgG1 production, which is in agreement with the work of Pasare and Medzhitov [16]. In addition, our data confirm previous findings showing that sensitization to OVA with alum adjuvant induced high levels of IgG1 that were significantly decreased in alum-free sensitization [18]. Regarding IgG2a/c isotype, our results clearly showed that irrespective of the usage or not of alum adjuvant, CpG augmented significantly OVA-specific IgG2c reinforcing the notion of the potentiating role of TLR signaling for IgG2a/c production.

It was previously shown that CpG induces murine B cells to proliferate and secrete immunoglobulin in vitro and in vivo [19]. Indeed, B cells express TLRs and respond to TLR agonists by differentiating into antibody-secreting cells [20]. *In vitro* experiments with purified B cells showed that CpG inhibits IgG1 and IgE switching induced by IL-4 and CD40 signaling [13]. This effect correlated with the expression of T-bet mRNA on purified B cells [13]. Another study showed CpG redirects Ig isotype production by regulating the specificity of class switch recombination directly on B cells in a manner critically dependent on TLR9 and myeloid differentiation molecule 88 (MyD88) expression [21]. However, the requirement of TLR-MyD88 signaling in IgG isotype class switching induced *in vivo* by sensitizations with different adjuvants indicated that MyD88 pathway is either essential or dispensable for enhanced IgG isotype production [16]. Interestingly, it was shown that for regulation of IgG2c isotype by CpG, that is encoded by IgH-1a haplotype [22], the requirement of *Myd88* expression on B-cells or dendritic cells (DCs) and/or other cell types varies according to the type or physical form of CpG formulation used [23–25]. We found that *Myd88* expression is fundamental for down regulation of IgE production by CpG, but *Myd88* expression on B cells was not necessary for CpG-induced IgE inhibition. In the same vein, enhancement of IgG2c switching by CpG did not require MyD88 molecule on B cells. Our results obtained *in vivo* with IgE regulations stand in contrast with experiments performed *in vitro* where CpG inhibited IgE switching induced by IL-4 and CD40 signaling [13] or with a study showing that CpG redirected Ig isotype production towards IgG isotypes by regulating the specificity of class-switch recombination directly on B cells in a manner critically dependent upon TLR9 and MyD88 expression [21] and with studies that suggested the requirement of direct B-cell stimulation by TLR ligands performed in reconstituted B-cell deficient mice with MyD88-deficient B lymphocytes [14]. Our results clearly indicated that CpG signaling through MyD88 molecule expressed on CD11c-positive putative DCs is necessary and sufficient for IgE inhibition and enhancement of IgG2c production *in vivo.* Pioneering studies of IgG2a/c regulation reported that all viral infections introduce a unique bias in the subclass selection process that makes IgG2a the predominant antiviral IgG antibody in the mouse in contrast to IgG1 predominance when soluble proteins are used for sensitization [26]. Recent studies with virus like particles (VLPs) or soluble antigens with TLRs agonists clarified the requirement of *Myd88* expression on B cells or DCs and/or other cells for IgG isotypes antibody production [25,27]. Notably, *Myd88* expression on B cells was critical for the production of high levels of IgG2a/c or IgG2b isotypes when VLPs containing TLR9 or TLR7 ligand or inactivated virus were used for immunizations [25,27]. In contrast and in line with our findings, it was shown that TLR-MyD88 signaling on DCs, but not on B-cells, was required for augmented production of IgG isotypes to a soluble protein either mixed with a soluble TLR9 ligand or chemically conjugated to it [25]. However, the DCs-dependency for augmented switching towards IgG2c isotype was lost when the physical form of CpG was changed to a more aggregated form [25]. These findings indicate that the adjuvant used, the site where sensitization is performed and the physical nature of the antigen determine the role of different cell types expressing *Myd88* on the enhancement of IgG production. In our model,

up-regulation of IgG2c production clearly required *Myd88* expression on DCs. Reports regarding the effect of CpG on IgE regulation *in vivo* and anaphylaxis are lacking. We found that sensitization to OVA with CpG consistently inhibited the production of OVA-specific IgE and/or anaphylactic antibodies by a mechanisms that was dependent on MyD88 expression on CD11c-positive putative DCs. Another intriguing finding was the fact that that CpG regulation of IgE or IgG2a/c isotype switching was independent of type I or type II interferon receptors. Our findings with IgE regulation corroborate previous work showing that type 1 cytokines are dispensable for the suppression of Th2-like immune responses by CpG [28]. In addition our data support the notion that IgG2a antibody production might proceed independently of type I IFN or of type II interferons as previously reported [29]. We speculate that regulation of class-switching by CpG might be mediated by the redundant effect of different types of inflammatory cytokines or by lipid inflammatory mediators [30].

The results obtained with sensitization to OVA with OVA-pulsed BM-DCs confirmed our results showing that *Myd88* expression on DCs is necessary and sufficient for CpG-mediated inhibition of IgE production. However, the CpG regulation of IgG1 or IgG2c isotypes in sensitization with OVA-pulsed DCs appears to be a much more complex process since we could not detect substantial production of IgG1 or IgG2c antibodies with OVA-primed DCs. Since IgG1 production was negligible in mice sensitized with OVA-primed DCs we suggest that induction IgE antibodies with OVA-pulsed DCs did not result from sequential switching from IgG1-positive B cells and as such the produced IgE antibodies probably did not suffer affinity maturation [31].

All in all, we demonstrate that DCs are the target cells governing *in vivo* regulation of IgE and IgG2c antibodies induced by sensitization to OVA with alum-based CpG formulation (see graphical abstract). Our findings might pave the way for the rational use of anti-allergic or anti-microbial vaccine formulations.

Author Contributions: Conceptualization, Ricardo Wesley Alberca-Custódio, Luciana Mirotti and Eliane Gomes; Data curation, Luciana Mirotti; Formal analysis, Ricardo Wesley Alberca-Custódio; Investigation, Ricardo Wesley Alberca-Custódio, Luciana Mirotti, Eliane Gomes, Fernanda Peixoto Barbosa Nunes, Raquel Souza Vieira and Rafael Ribeiro Almeida; Methodology, Ricardo Wesley Alberca-Custódio, Eliane Gomes, Raquel Souza Vieira and Rafael Ribeiro Almeida; Project administration, Ricardo Wesley Alberca-Custódio and Momtchilo Russo; Resources, Momtchilo Russo; Supervision, Luis Graça, Niels Olsen Saraiva Câmara and Momtchilo Russo; Visualization, Momtchilo Russo; Writing – original draft, Ricardo Wesley Alberca-Custódio and Momtchilo Russo; Writing – review & editing, Ricardo Wesley Alberca-Custódio, Luis Graça, Niels Olsen Saraiva Câmara and Momtchilo Russo.

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Legends to the Figures

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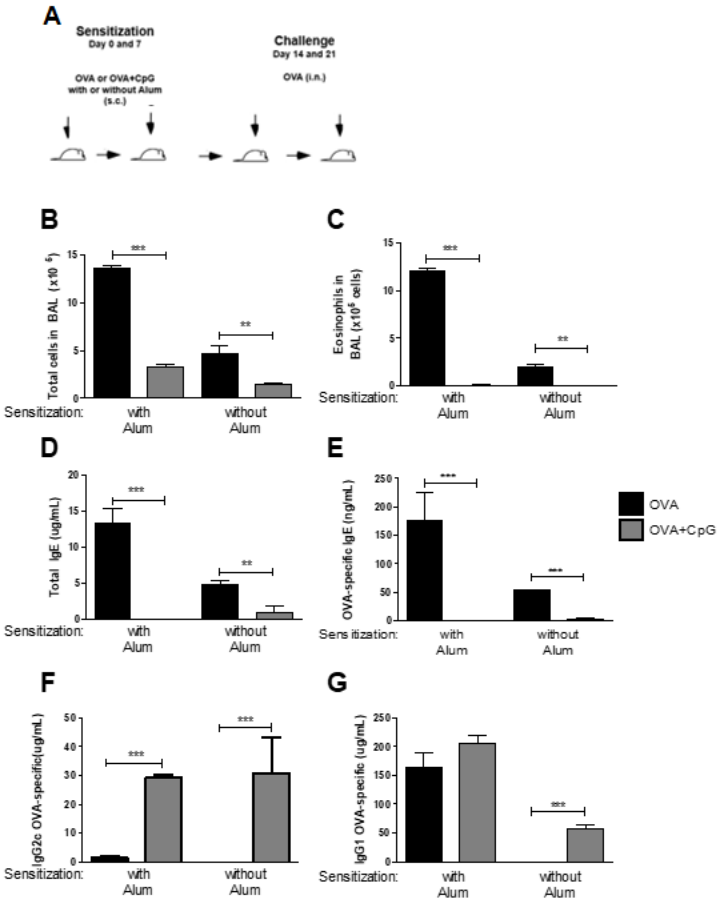

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335 **FIGURE 1: CpG inhibits IgE and enhances IgG production. C57BL/6 wild-type (WT) mice were**
336 **sensitized subcutaneously with ovalbumin (OVA) or OVA plus CpG (OVA+CpG) with or**
337 **without alum adjuvan on days 0 and 7 and challenged intranasally with OVA on days 14 and 21.**
338 **Experiments were performed on day 22.** (A) **Schematic experimental protocols. Numbers of** (B)
339 **Total Cells and** (C) **Eosinophils in BAL. Serum levels of** (D) **total IgE,** (E) **OVA-specific IgE,** (F)
340 **OVA-specific IgG2c or (G) OVA-specific IgG1. OVA groups (*n* = 5) and OVA+CpG groups (*n* = 5).**
341 **Values represent the mean ± SD and are representative of two independent experiments.**
342 **One-way ANOVA: **$p < 0.01$; ***$p < 0.001$.**

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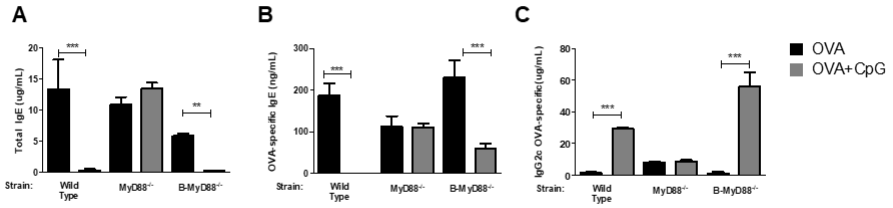

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345 **FIGURE 2: Myd88 expression on B-cells is dispensable for CpG-mediated inhibition of IgE**
346 **production. C57BL/6 wild-type (WT) or mice lacking total Myd88 expression (MyD88$^{-/-}$) or on B**
347 **lymphocytes (B-MyD88$^{-/-}$) mice were subcutaneously sensitized with ovalbumin (OVA) or OVA**
348 **plus CpG using alum as adjuvant on days 0 and 7 and challenged intranaselly with OVA on days**
349 **14 and 21. Experiments were performed on day 22. Serum levels of** (A) **Total IgE,** (B)
350 **OVA-specific IgE and** (C) **OVA-specific IgG2c. OVA groups (*n* = 5) and OVA+CpG groups (*n* = 5).**

Values represent the mean ± SD and are representative of two independent experiments. One-way ANOVA:** $p < 0.01$; *** $p < 0.001$.

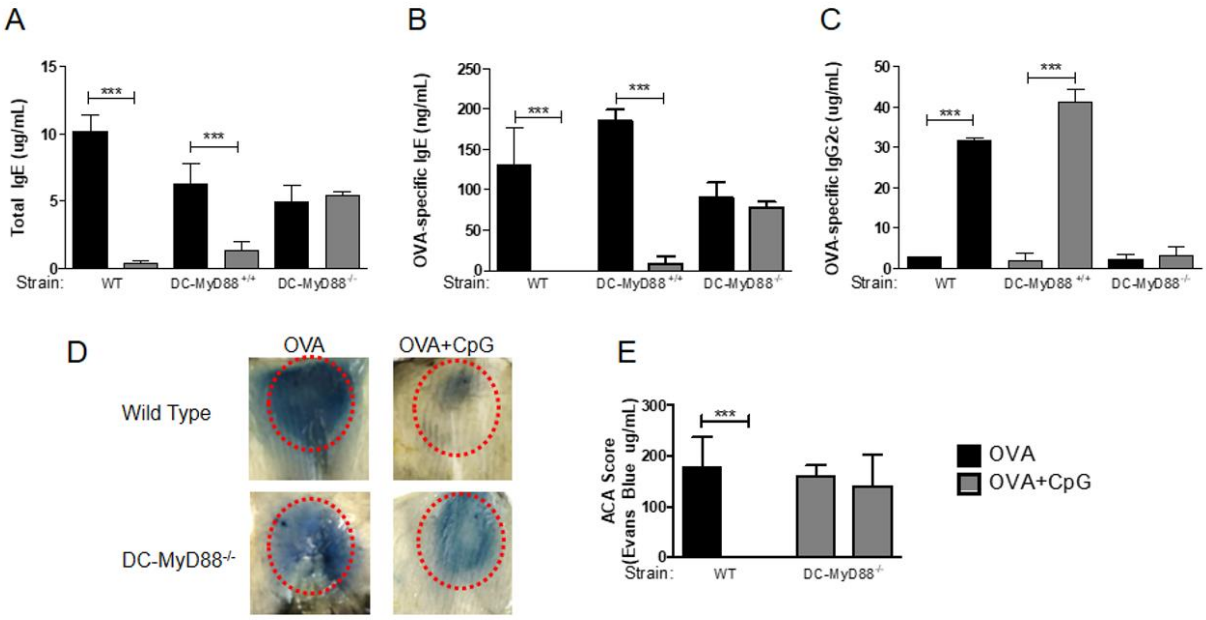

FIGURE 3: Myd88 expression on CD11c-positive cells mediates CpG-induced regulation of IgE and IgG2c production. C57BL/6 wild-type (WT) or mice lacking total Myd88 expression on CD11c-positive dendritic cells (DC-MyD88$^{-/-}$) or their control littermates (DC-MyD88$^{+/+}$) mice were subcutaneously sensitized with ovalbumin (OVA) or OVA plus CpG using alum as adjuvant on days 0 and 7 and challenged intranasally with OVA on days 14 and 21. Experiments were performed on day 22. Serum levels of (A) total IgE, (B) OVA-specific IgE or (C) OVA-specific IgG2c. Active cutaneous anaphylaxis (ACA) assay measured by Evans blue dye extravasation upon intradermal OVA injection was determined on day 21. (D) Representative skin pictures of Evans blue extravasation and (E) ACA score determined by measuring Evans blue extracted from the tissue. Experiments performed in 129 WT strain or mice lacking type I interferon receptor (IFNAR$^{-/-}$) or type II interferon receptor (IFNGR$^{-/-}$) on 129 background used the same protocol of OVA sensitization and challenge. Experiments were performed on day 22. Serum levels of (F) total IgE, (G) OVA-specific IgE or (H) OVA-specific IgG2c. OVA groups ($n = 5$) and OVA+CpG groups ($n = 5$). Values represent the mean ± SD and are representative of two independent experiments. One-way ANOVA: *** $p < 0.001$.

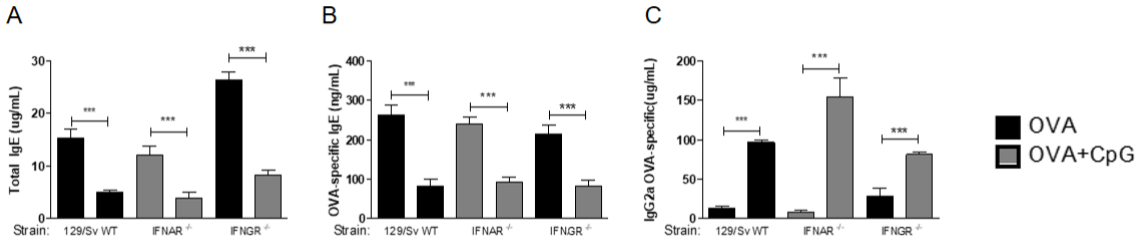

FIGURE 4: CpG-induced inhibition of IgE and augmented IgG2a production is independent of type I or type II interferon receptors. 129 WT strain or mice lacking type I interferon receptor

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(IFNAR$^{-/-}$) or type II interferon receptor (IFNGR$^{-/-}$) on 129 background used the same protocol of OVA sensitization and challenge. Experiments were performed on day 22. Serum levels of (A) total IgE, (B) OVA-specific IgE or (C) OVA-specific IgG2c. OVA groups ($n = 5$) and OVA+CpG groups ($n = 5$). Values represent the mean ± SD and are representative of two independent experiments. One-way ANOVA: ***$p < 0.001$.

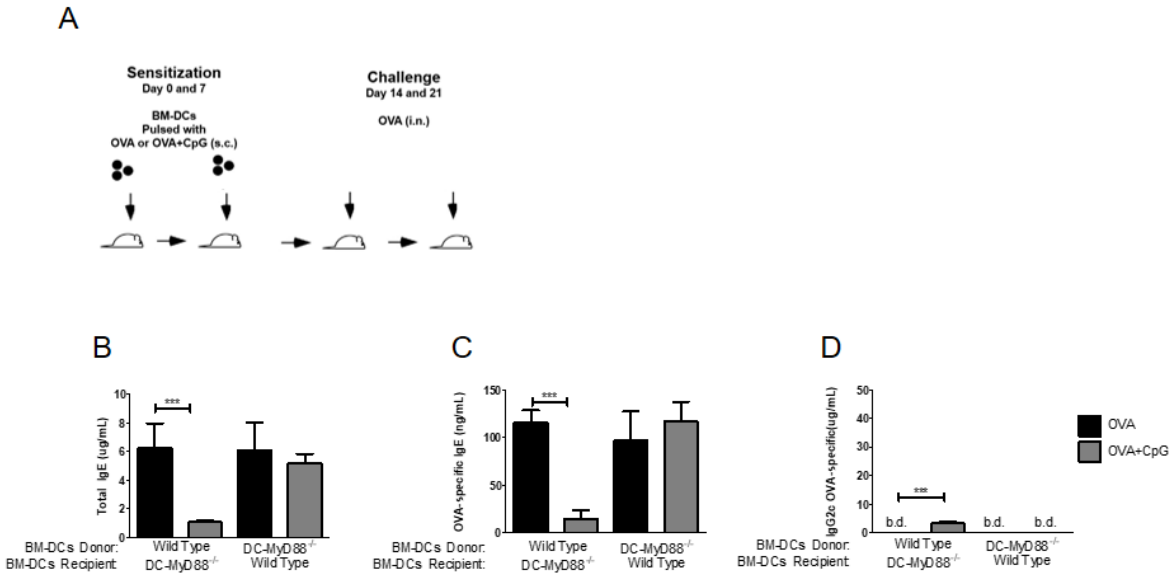
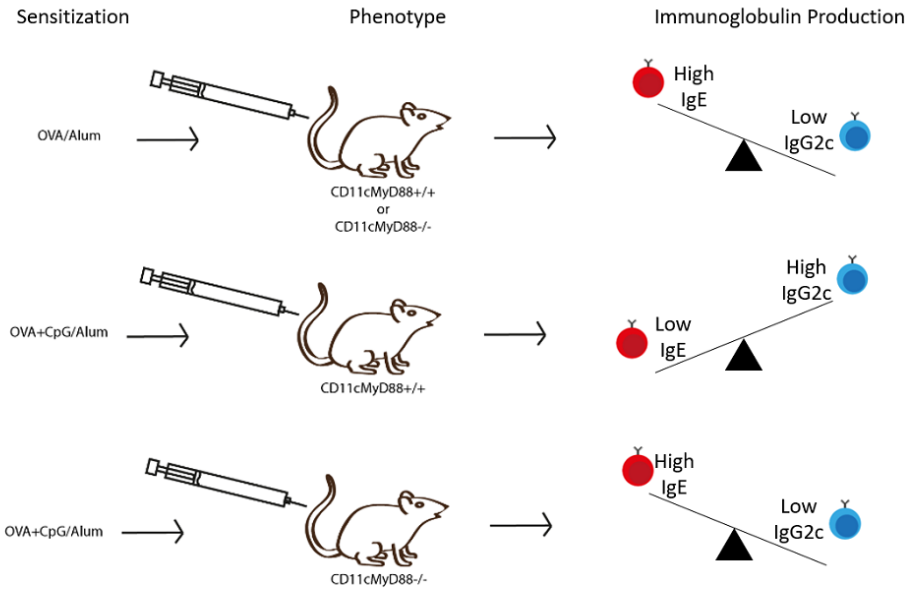


FIGURE 5: Myd88 expression on CD11c-positive dendritic cells is essential for CpG-induced suppression of IgE production. Bone marrow-derived dendritic cells (BM-DCs) obtained from C57BL/6 wild type (WT) or mice lacking Myd88 on CD11c-positive dendritic cells (DC-MyD88$^{-/-}$) donors were pulsed with OVA or OVA plus CpG and injected subcutaneously on days 0 and 7 respectively to DC-MyD88$^{-/-}$ or WT recipient mice. On days 14 and 21 recipient mice were challenged intranasally with OVA Experiments were performed on day 22. (A) Schematic experimental protocol. Serum levels of (B) total IgE, (C) OVA-specific IgE or (D) OVA-specific IgG2c. OVA groups ($n = 5$) and OVA+CpG groups ($n = 5$). Values represent the mean ± SD and are representative of two independent experiments. b.d.- bellow detection level and One-way ANOVA: ***$p < 0.001$, different from respective OVA group



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393 **Graphical Abstract: Mice sensitized with OVA/Alum develop a high production of IgE and low**
394 **production of IgG2c antibodies whereas in mice sensitized with OVA+CpG/Alum IgE**
395 **production is abrogated and OVA-specific IgG2c antibody production increases. However, IgE**
396 **inhibition and augmented IgG2c production does not occur after sensitization with**
397 **OVA+CpG/Alum in mice lacking Myd88 gene expression on CD11c-positive dendritic cells**
398 **(DC-MyD88$^{-/-}$).**

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