



Study of the allergenic potential of *Bacillus thuringiensis* Cry1Ac toxin following intra-gastric administration in a murine model of food-allergy

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ABSTRACT

Cry1Ac toxin, from *Bacillus thuringiensis*, is widely used as a biopesticide and expressed in genetically modified (GM) plants used for human and animal consumption. Since Cry1Ac is also immunogenic and able to activate macrophages, it is crucial to thoroughly evaluate the immunological effects elicited after intra-gastric administration. The allergenic potential of purified Cry1Ac was assessed and compared with that induced in a murine model of food-allergy to ovalbumin (OVA), in which animals are sensitized with the adjuvant Cholera toxin (CT). Mice were weekly intragastrically administered with: i) vehicle phosphate-buffered saline (PBS), ii) OVA, iii) OVA plus CT iv) Cry1Ac or v) OVA plus Cry1Ac. Seven weeks after, mice were intragastrically challenged and allergic reactions along with diverse allergy related immunological parameters were evaluated at systemic and intestinal level. The groups immunized with, Cry1Ac, OVA/Cry1Ac or OVA/CT developed moderate allergic reactions, induced significant IgE response and increased frequencies of intestinal granulocytes, IgE+ eosinophils and IgE+ lymphocytes. These same groups also showed colonic lymphoid hyperplasia, notably in humans, this has been associated with food allergy and intestinal inflammation. Although the adjuvant and allergenic potential of CT were higher than the effects of Cry1Ac, the results show that applied intra-gastrically at 50 µg doses, Cry1Ac is immunogenic, moderately allergenic and able to provoke intestinal lymphoid hyperplasia. Moreover, Cry1Ac is also able to induce anaphylaxis, since when mice were intragastrically sensitized with increasing doses of Cry1Ac, with every dose tested, a significant drop in rectal temperature was recorded after intravenous challenge.

1. Introduction

Cry proteins are produced as proteinaceous crystals during the sporulation phase by *Bacillus thuringiensis* (Bt). These proteins are widely used as biopesticides; also they demonstrate a high solubility, stability in alkaline pH and resistance to proteolysis [1]. After crystal ingestion by susceptible insects, Cry protoxin is solubilized and proteolytically activated by midgut proteases to form a toxic fragment of 65–70 kDa. The activated toxin then binds to specific receptors present on the apical brush border of the midgut epithelium, which provokes formation of a lytic pore, inducing a complex cellular response that disrupts the cell integrity and transepithelial potential difference, ultimately leading to cell lysis and larval death [2,3].

Genetically modified (GM) plants that express the Cry insecticidal toxin as a highly effective means of insect control are currently being produced and a variety of Bt plants have been commercialized in many countries. These plants can express one or several different Cry toxins [3]. Several toxicological studies conducted in animals have evaluated Bt plants; these analyses have included laboratory tests such as, blood chemistry, evaluation of food consumption, body and organ weight and pathological analysis including the presence of tumors. The majority of these studies indicate that GM food containing Cry toxins are safe for human and animal consumption as found no abnormalities in the parameters evaluated, toxicity or death associated with Cry proteins [4–10].

Notwithstanding, the potential adverse immune effects of Cry toxins

Abbreviations: Bt, *Bacillus thuringiensis*; BSA, bovine serum albumin; CT, cholera toxin; Cry1Ac, Cry1Ac toxin; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FCS, fetal calf serum; GM, genetically modified; H&E, hematoxylin and eosin; LI, large intestine; MLN, mesenteric lymphoid node; mAbs, monoclonal antibody; mMCP-1, mouse mast cell protease-1; NLH, nodular lymphoid hyperplasia; IBD, inflammatory bowel disease; CeD, celiac disease; OVA, ovalbumin; PBS, phosphate-buffered saline; PP, Peyer's patch; rpm, revolutions per minute; SI, small intestine

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is still a public health concern, due to the significant raise in the incidence of allergy and inflammatory bowel diseases recorded particularly in industrialized countries, over the past few decades [11]. Moreover, due to the immunological properties of Cry1Ac proteins, such as mucosal and systemic immunogenicity and adjuvanticity [12–19]; along with its capacity to activate macrophages [20,21], their potential ability to provoke either allergic or inflammatory responses has been suggested, but remains to be proved [22]. Importantly, we have recently demonstrated that Cry1Ac protoxin and toxin are both able to activate macrophages and induce the activation of MAPKs pathways [23,24], further reinforcing the need for a deeper evaluation of their *in vivo* immunological effects in mammals.

A few studies have evaluated the immunogenicity and potential allergenicity of Cry toxins, after intra-gastric administration; these have been centered just in Cry1Ab purified toxin (a protein homologous in structure to Cry1Ac) and in maize MON810 (expressing Cry1Ab). Although some studies have supported its immunogenicity, but not its allergenicity, the approaches employed were limited, such as in the study of Adel et al. 2011 in BALB/c mice, in which was found that intraperitoneal administration of purified toxin and maize MON810 induced serum IgG and IgA production, but did not induce IgE antibody response. However, they did not detect significant serum antibody responses after intra-gastric administration of Cry1Ab, but nor with any of the proteins tested. These negative results may be due to the inadequate immunization scheme used, which consisted on just two administrations with 1 or 100 µg, thus differs considerably from the schemes used in models to evaluate food allergy which include several repeated immunizations. Moreover, the study included neither any analysis of intestinal immune response [4]. In a recent study of Andreassen et al., no evidence of immunogenicity or allergenicity of Cry1Ab was recorded after intra-gastric administrations of low dose of the toxin (10 µg) and intraperitoneal challenges [25], however a comprehensive evaluation of the intestinal immune response was not performed.

In the present study we determined the allergenic potential and immunological effects, of purified Cry1Ac toxin after intra-gastric administration to mice. We selected and adapted an administration scheme similar to that used in mouse models of food allergy to be able to compare the immunological effects of Cry1Ac [26,27]. In this model, mice are intragastrically sensitized to ovalbumin (OVA) in the presence of the Th2-type mucosal adjuvant cholera toxin (CT), or tolerized by intra-gastric administrations of OVA alone; while the antigenic challenge is also administered by intra-gastric route. Thus the model more realistically resembles the antigen entry route occurring in human food allergy, in contrast to other allergy models that use either systemic sensitization or challenges [28]. We performed a wide evaluation of parameters related with intestinal and systemic allergic response such as: i) allergic reactions, ii) specific analysis of IgG1, IgG2a, IgE antibody responses in serum and intestinal lavages, iii) quantification of granulocytes and B220+ IgE+ lymphocytes in spleen, mesenteric lymph node (MLN), Peyer's patches (PP) and intestinal lamina propria cells; iv) analysis of cytokine profiles and v) intestinal histopathological analysis. Overall the results indicate the potential of Cry1Ac toxin to elicit moderate allergy-related responses, when administered via intra-gastric route.

Moreover, Cry1Ac is also able to induce anaphylaxis, as when mice were intragastrically sensitized with increasing doses of Cry1Ac, with every dose tested, a significant drop in rectal temperature was recorded after intravenous challenge further confirming the potential allergenicity of Cry1Ac.

2. Materials and methods

2.1. Animals

Female BALB/c mice (6–8 week-old) were used throughout this

study. These mice were provided with sterile food (2019 Teklad Global 19% Protein Extruded Rodent Diet; Harlan Laboratories Inc. Indianapolis, IN) and water *ad libitum*, at the FES Iztacala, Universidad Nacional Autónoma de México animal house facility in accordance with institutional and national official guidelines (NOM-062-ZOO-1999) for the use and care of laboratory animals. For the majority of the results shown (Figs. 1–5) two to three independent experiments were performed with 4–5 individuals per group, the number (n) of mice used per group is indicated in each figure legend.

2.2. Recombinant Cry1Ac toxin

Recombinant Cry1Ac protoxin was purified from IPTG induced *E. coli* JM103 (Pos900) cultures [29]. Protoxin was solubilized, and the toxin was activated with trypsin as previously described [19]. Endotoxin levels of Cry1Ac toxin were tested using E-toxate kit (Sigma Chemical Co., St. Louis, USA) and were found to be below 0.1 EU/mL. The protein was then treated with a polymyxin resin (Bio-Rad, Hercules CA, USA) to remove any possible endotoxin remnants, examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the total protein concentration was determined by the Bradford method.

2.3. Immunizations

The sensitization protocol used was adapted from a described model to establish food allergy to OVA [26], BALB/c mice of 6–8 weeks old female received intra-gastric administrations. In the allergy positive group mice were sensitized with OVA (Worthington, Lakewood, USA) plus CT (Sigma Chemical Co., St. Louis, USA) while in the negative control tolerized group mice received OVA alone. In this model [26], as in the majority of CT-induced food allergy models high antigen doses are intra-gastrically administered (i.e. 20 mg of OVA), but since our main purpose was to evaluate the allergenicity of Cry1Ac, two distinct doses of OVA (50 µg and 5 mg) were proved in both control groups (OVA alone and OVA plus CT). The lower dose of OVA was initially considered to make it comparable to the dose chosen of Cry1Ac (50 µg). The expected allergy related parameters were attained in the OVA plus CT positive groups, (Supplementary Fig. 1) but these were more marked and consistent with the higher OVA dose than with the lower dose, therefore only the 5 mg dose was used in the subsequent assays.

The groups of mice were intragastrically administered, once a week for seven weeks, using a ball-ended mouse feeding needle, (in 200 µL volumes diluted in PBS) (Fig. 1A), with one of the following treatments: i) vehicle PBS; ii) 50 µg or 5 mg of OVA tolerized control; iii) 50 µg or 5 mg of OVA plus 50 µg of CT as positive allergy control; iv) 50 µg or 5 mg of OVA plus 50 µg of Cry1Ac and v) 50 µg of Cry1Ac alone and v), the last group were test groups used to evaluate if Cry1Ac could induce sensitization to itself respectively to OVA). On day 49, mice received intra-gastric. Challenge with a higher OVA antigen doses, (i.e. 300 µg of OVA for OVA 50 µg groups and 20 mg of OVA for OVA 5 mg groups), or with 300 µg of Cry1Ac (in mice of group Cry1Ac alone). Mice were sacrificed 1 h after the intra-gastric challenge at day 49 (Fig. 1A).

The dose used of 50 µg Cry1Ac was chosen on basis to a dose-response preliminary study in which four distinct doses were tested (10, 50, 100 and 200 µg), to evaluate the immunogenicity via the intra-gastric route, this dose proved to induce significant anti-Cry1Ac serum and intestinal antibody responses using a 3 immunization schedule (Data not show). This dose, also has been previously proved to be immunogenic via intranasal route [19]. The OVA stock solution was also treated with polymyxin resin to remove any possible endotoxin contamination.

2.4. Evaluation of allergic reactions

Allergic reactions were assessed by measuring changes in the body

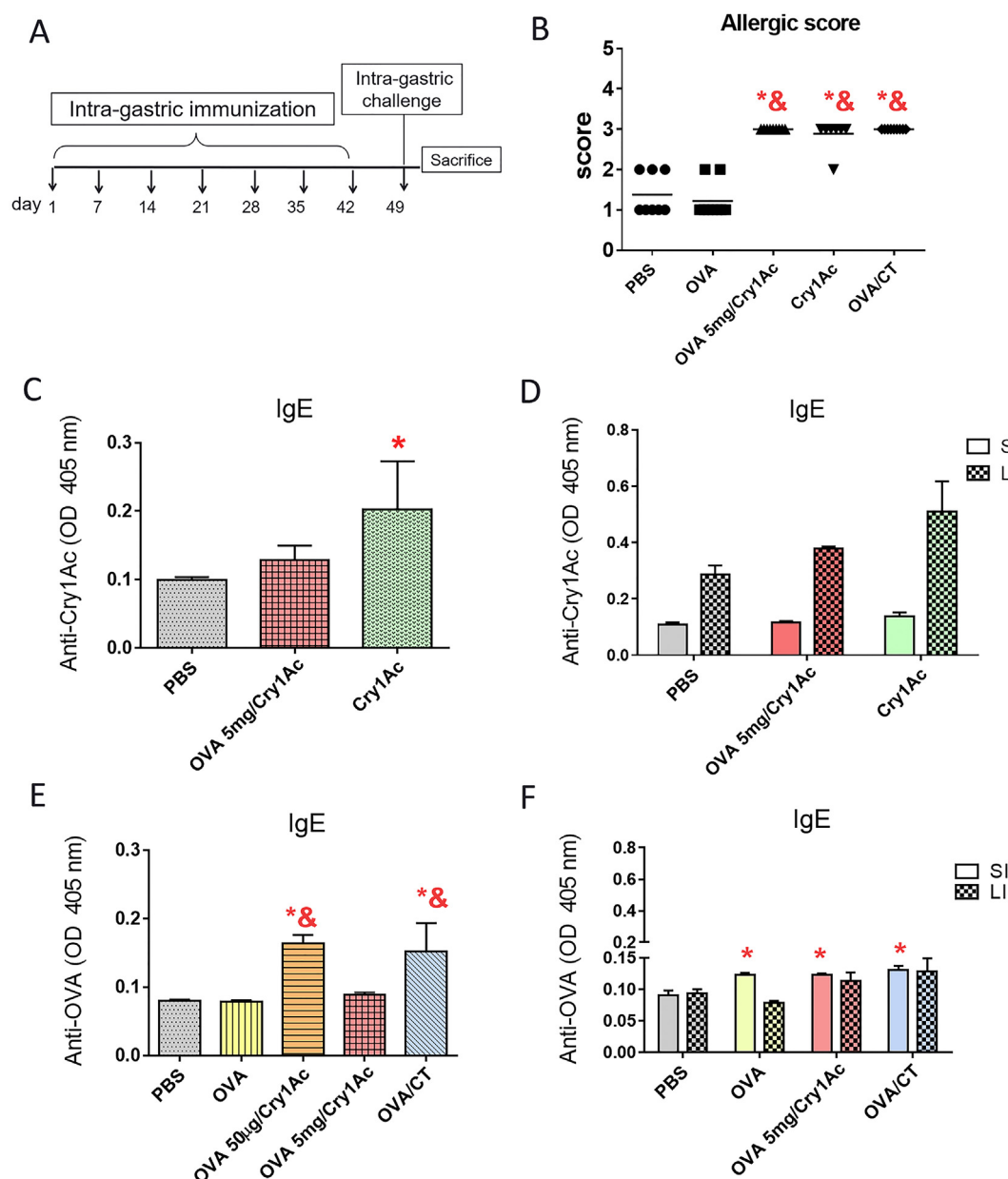


Fig. 1. Protocol of sensitization by intra-gastric route and allergic response. (A) BALB/c mice were intra-gastrically administered once a week for 7 weeks with one of the following treatments: phosphate buffered saline (PBS); ovalbumin (OVA); OVA plus cholera toxin (CT). Cry1Ac alone or OVA plus Cry1Ac. At day 49, mice were challenged intra-gastrically with OVA or Cry1Ac as detailed in methodology and sacrificed 1 h after. (B) The allergic reactions were determined immediately after intra-gastric challenge, over a 15 min period, based on the following scoring system: 0- no signs; 1- scratching around nose, ears and head less than 10 times in 15 min; 2- scratching 10 times in 15 min; 3- scratching more than 10 times, puffiness around the eyes and mouth, diarrhea, pilar erecti in 15 min; 4- reduced physical activity and/or decreased activity with increased respiratory rate; 5- death. (C) the graphic shows the Cry1Ac-specific IgE antibody response in serum, (D) Cry1Ac-specific IgE antibody response in intestine small intestine (SI) and large intestine LI, (E) shows the response of OVA-specific IgE in serum and (F) response of OVA-specific IgE antibody response in intestine SI (open bars) and LI (close bars). The results shown in A–E were obtained from independent experiments performed using 4 mice per group (n = 8 to 12). Significant differences ($P < 0.05$) are indicated as follows: * versus PBS and & versus OVA.

temperature recorded before and 30 min after the intra-gastric challenge and during 15 min post-challenge the signs of allergic reactions were recorded and monitored in a blinded fashion by two independent researchers. Allergic scores were subsequently graded adhering to an pre-established 0–5 scale [26] consisting of the following signs: 0- no signs; 1- scratching around nose, ears and head less than 10 times for 15 min; 2- scratching 10 times for 15 min; 3- scratching more than 10 times, puffiness around the eyes and mouth, diarrhea, pilar erecti for 15 min; 4- reduced physical activity and/or decreased activity with increased respiratory rate; 5- death.

2.5. Anaphylaxis

Although anaphylaxis can be achieved following intra-gastric challenge to some allergenic proteins such as peanut it is not attained in BALB/C mice sensitized and challenged intra-gastrically to OVA [30], while intravenous challenges protocol had proved to be effective to induce hypothermia shock responses [31]. So, in additional experimental groups one day after receiving an intra-gastric challenge, were carried out intravenous challenges with 100 µg of OVA (in PBS, OVA, OVA plus Cry1Ac and OVA plus CT groups); or with 100 µg of Cry1Ac (in mice intra-gastrically administered with PBS or with increasing

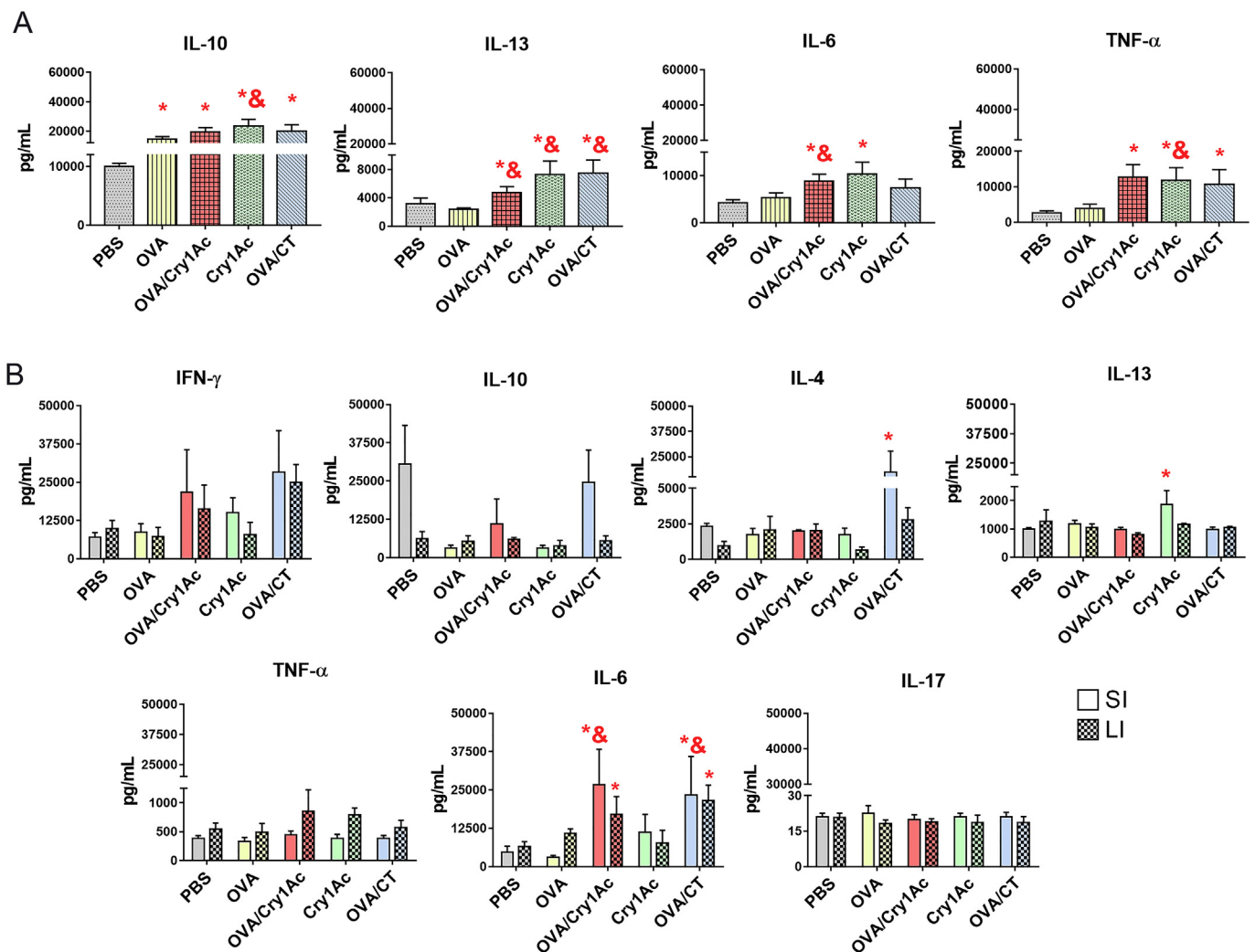


Fig. 2. Intra-gastric administration of Cry1Ac induces a mixed cytokine profile. Cytokines were quantified by ELISA in serum samples (A) IL-10, IL-13, IL-6 and TNF- α cytokines and (B) IFN- γ , IL-10, IL-4, IL-13, TNF- α cytokines were quantified in intestinal lavages and represented in the graphics. The results are expressed as mean $\pm$ SEM (n = 8 to 12 mice per group), significant differences ($P < 0.05$) are indicated as follows: * versus PBS and & versus OVA.

doses (10, 50, 100 and 200 μ g) of Cry1Ac (Fig. 5A). Temperature changes were monitored using rectal probe thermometer and significant temperature drop was used as a measure of anaphylaxis.

2.6. Determination of anti-OVA and anti-Cry1Ac antibody levels in serum and intestinal lavages

Serum samples were obtained from blood extracted by cardiac puncture and large intestine (LI) and small intestine (SI) lavages as previously described [32] from sacrificed mice. Anti-OVA and anti-Cry1Ac antibody responses were measured by indirect enzyme-linked immunosorbent assay (ELISA). Plates were coated overnight at 4 $^{\circ}$ C with 100 μ L of Cry1Ac (10 μ g/mL) or OVA (10 μ g/mL) in carbonate-bicarbonate buffer (0.1 M Na₂CO₃-NaHCO₃ pH 9.6). Non-specific binding sites were blocked with 0.05% PBS-Tween 20/3% BSA for serum and 0.05% PBS-Tween 20/10% non-fat milk for intestinal lavages samples for 2 h at 4 $^{\circ}$ C. Serial dilutions of samples were incubated over night at 4 $^{\circ}$ C in volumes of 100 μ L per well. These started at 1:100 for serum, while for SI and LI lavages were 1:2 diluted with 20% non-fat milk. Peroxidase-conjugated anti-mouse anti-IgG1, anti-IgG2a (Invitrogen, California, USA), and anti-IgA (Life technologies, California, USA) were used at a dilution of 1:1000 and biotinylated anti-mouse anti-IgE (eBioscience, San Diego, USA) used at 1:500. The substrate solution was ABTS 2, 2'-azino-di (3-ethyl-benzthiazoline-6-sulphonate

with H₂O₂ (0.01%). The absorbance was measured at 405 nm using a microplate reader (Biotek ELx 800).

2.7. Spleen, mesenteric lymphoid node and Peyer's patch leukocytes

The Spleen, mesenteric lymphoid node (MLN) and Peyer's patch (PP) were removed and disaggregated through a nylon mesh filter with RPMI-1640 medium supplement with 5% fetal calf serum (FCS) and centrifuged at 1500 rpm for 5 min at 4 $^{\circ}$ C. In spleen, the erythrocytes were lysed with (NH₄Cl 150 mM/L, Tris-HCl 100 mM pH 7.4) for 5 min, and washed with PBS; the leukocyte pellets were then suspended in 0.5% BSA/PBS and stained with fluorochrome-labeled mAbs as described [24].

2.8. Lamina propria leukocytes

The SI and LI were removed, separated and the lavages were collected. The intraepithelial and lamina propria cells were obtained from both intestines according to a previously published method [32] with some modifications, basically no-percoll gradients were used. After obtaining the intraepithelial cells, the intestines were incubated in an orbital shaker with 20 mL of RPMI-1640 and 60 U/mL of type IV collagenase (Sigma Chemical Co., St. Louis, USA) at 37 $^{\circ}$ C for 30 min at 120 rpm. The intestinal mucosa was then gently compressed and shaken

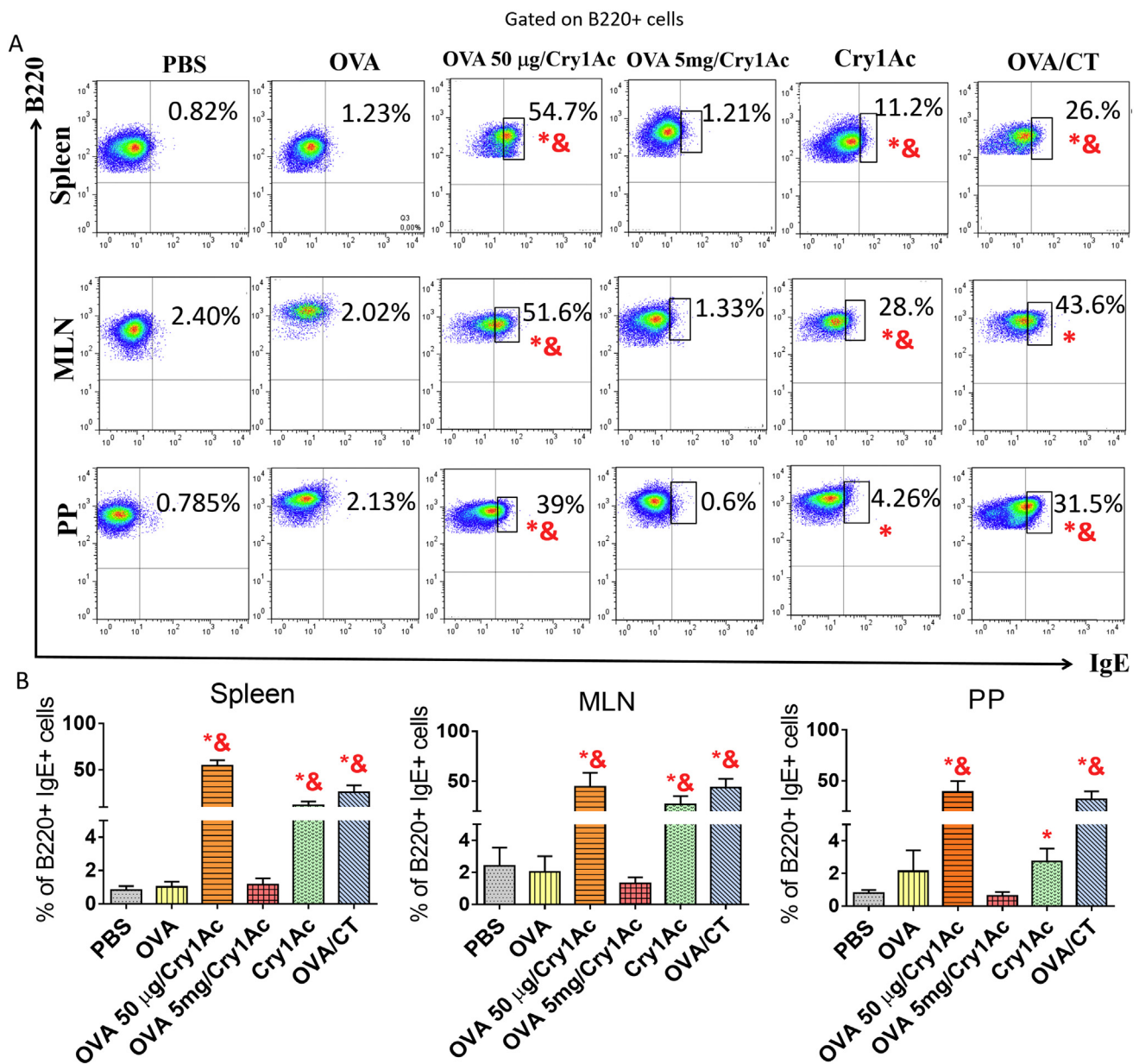


Fig. 3. The Cry1Ac protein increases the proportion of B220 + IgE + lymphocytes in spleen, mesenteric lymph node and Peyer's patches. Spleen, mesenteric lymph node (MLN) and Peyer's patches (PP) cells were obtained from BALB/c mice, treated as described in Fig. 1. The cells were stained with mAbs and analyzed by flow cytometry to determine the proportion of B220 + IgE + lymphocytes. (A) Representative dot plots of spleen, MLN and PP cells are shown for each group indicating the percentage of B220 + IgE + lymphocytes. (B) Bar plots show the mean values $\pm$ SEM of percentage of B220 + IgE + lymphocytes from each group. Data shown were obtained from independent experiments ($n = 8$ to 12 mice per group). Significant differences ($P < 0.05$) are indicated as follows: * versus PBS and & versus OVA.

to release the loosened cells and single cell suspensions containing lamina propria cells, were filtered, washed with RPMI-1640 medium with 5% fetal bovine serum (FBS) and centrifuged at 1500 rpm for 10 min at 4 °C. Finally, cells were suspended in 0.05% BSA/PBS and stained with fluorochrome labeled mAbs [33].

2.9. Levels of mast cell protease-1 mMCP-1 and cytokines

The mouse mast cell protease-1 (mMCP-1 also abbreviated as MCPT-1) levels were measured using an ELISA kit according to the manufacturer's directions (eBioscience, San Diego, USA) in serum samples 1:2 diluted. The levels of cytokines (IFN- γ , IL-10, IL-4, IL-13, IL-6, TNF- α) in serum samples and intestinal lavages, were determined by ELISA using commercial kits following the manufacturer's specifications (PEPROTECH, New Jersey, USA). Serum samples were diluted

1:4 and intestinal lavages were diluted 1:2 added in volumes of 100 μ L per well for quantification of cytokines.

2.10. Flow cytometry

The leukocytes obtained from spleen, MLN, PP and the intestinal lamina propria were blocked with antibodies to the Fc γ II/III receptor (anti-CD16/32). Cells were then incubated for 20 min at 4 °C with 25 μ L of the following 1:100 diluted monoclonal antibodies: IgE-FITC (BioLegend, San Diego, USA), B220-PECy5 (eBioscience, San Diego, USA), Siglec-F-PE (eBioscience, San Diego, USA) and Gr1-APC (BioLegend, San Diego, USA). The cells were washed with 0.05% BSA/PBS and suspended in 300 μ L of 1% paraformaldehyde. Relative fluorescence intensities were analyzed using a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA) and BD Cell Quest Pro v.5.1.1

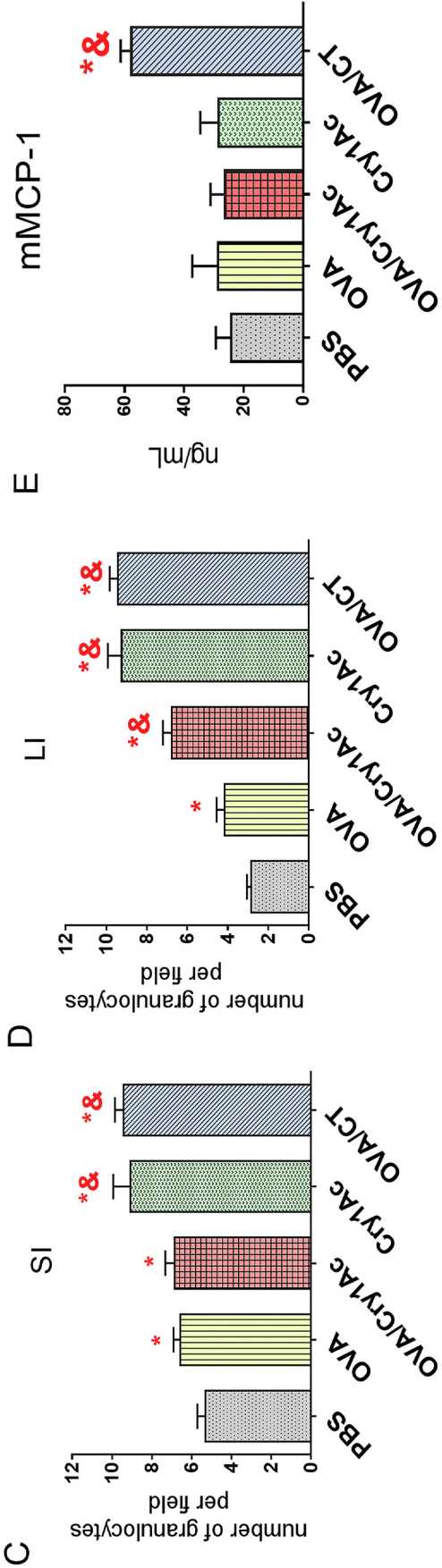
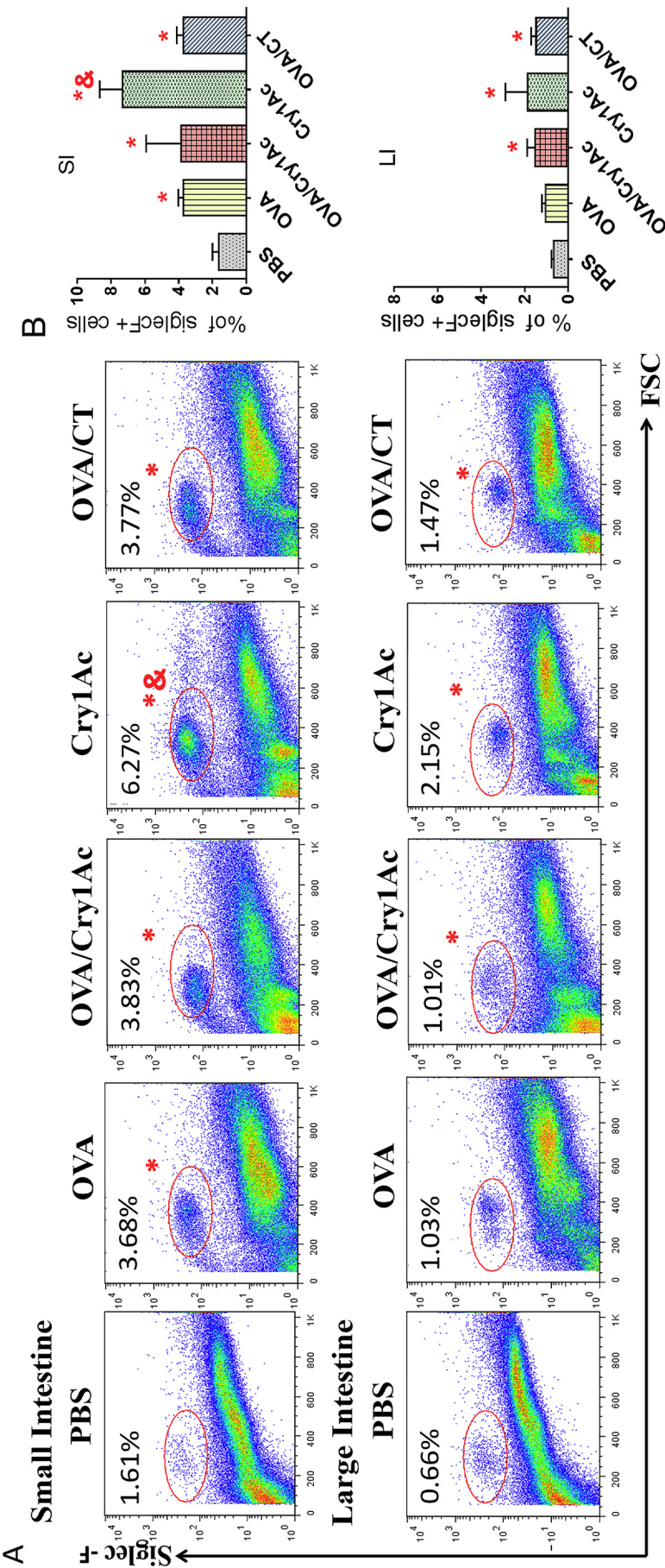


Fig. 4. Analysis of Siglec-F+ cells in lamina propria, granulocytes count in intestine and quantification of mMCP-1 in serum. Mice were treated as indicated in Fig. 1. Lamina propria cells were obtained from SI and LI and stained with mAbs to determine the proportion of Siglec-F+ cells (eosinophils) by flow cytometry. (A) Representative dot plots show the percentage of Siglec-F+ cells (indicated with circles) in each group. (B) Bar plots indicate the mean $\pm$ SEM corresponding to the percentage of Siglec-F+ cells in SI and LI. (C) SI tissue sections and (D) LI tissue sections were labeled with H&E stain and the number of total granulocytes was counted in each section. The graphs show the mean values $\pm$ SEM of the granulocytes count from twenty independent cross-sections (n = 5 mice per group). (E) Concentration of mouse mast cell protease mMCP-1 in serum (n = 8–12 mice per group). Results shown in (B and E) were obtained from independent experiments using 4 mice per group (n = 8–12 mice per group). Significant differences ($P < 0.05$) are indicated as follows: * versus PBS and & versus OVA.

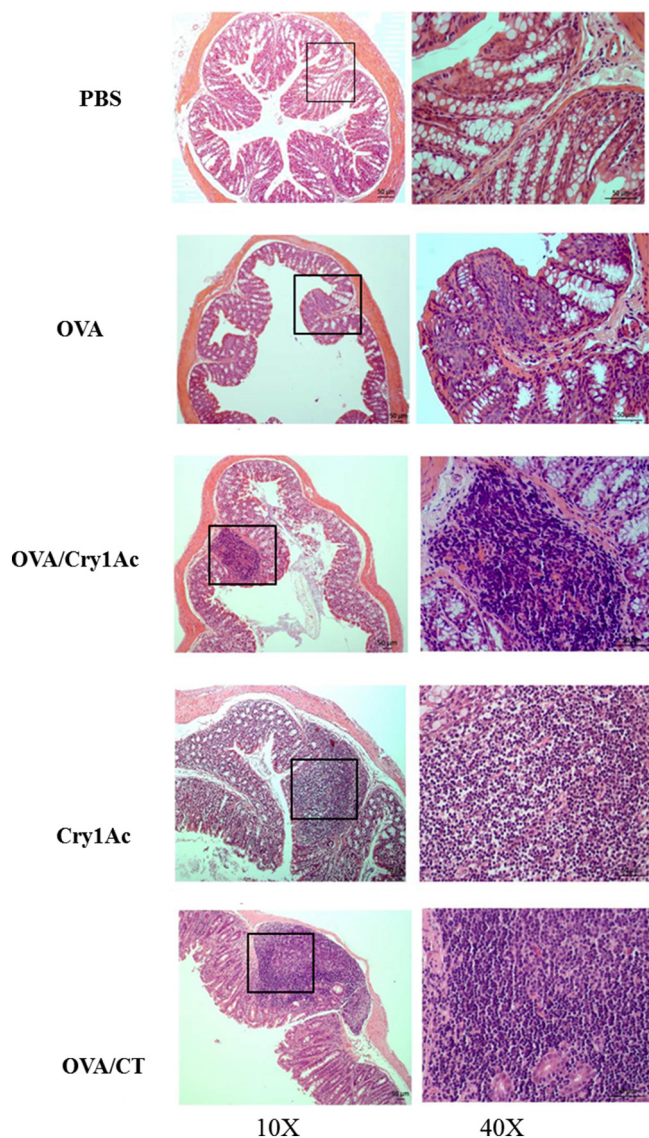


Fig. 5. Treatments with Cry1Ac induces intestinal lymphoid hyperplasia of the colon. Colon sections obtained from BALB/c mice treated as indicated in Fig. 1 were labeled with H&E stain for pathological analysis. The images are representative of colon sections (LI) from each treatment group ($n = 8$ to 12 mice per group). Intestinal lymphoid hyperplasia was induced by OVA/Cry1Ac, Cry1Ac and OVA/CT treatment.

software. One hundred thousand events were recorded; data were analyzed using the FlowJo 7.6 software.

2.11. Histological analysis of intestinal tissues

Intestinal segments 0.5–1 cm long were cut from duodenum, jejunum and ileum of SI and ascending, transverse and colon of LI tissues and were fixed in 4% paraformaldehyde. Tissue slices were dehydrated in graded alcohol and paraffin, cut into 3–6 μm cross-sections and stained with hematoxylin and eosin (H&E). Total granulocytes from twenty independent cross-sections per group were counted under a microscope (Axiovert Zeiss, New Orleans, USA) in SI and LI tissue sections. The pathological analysis of intestinal tissue sections was performed by expert pathologist services from the Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México.

2.12. Competition assays of binding of OVA and Cry1Ac to macrophage's cell surface

We performed competitions assays of OVA and Cry1Ac in RAW264.7 macrophages as described [21], cells were pre incubated at 4 °C in binding buffer (PBS with 1% BSA) with the unlabeled Cry1Ac (200 $\mu\text{g}/\text{mL}$) or OVA (1 mg/mL) for 30 min on ice. Then the labeled ligand OVA-FITC (3 $\mu\text{g}/\text{mL}$) was added and the incubation was continued for another 1 h on ice. The samples were fixed and analyzed by flow cytometry.

2.13. Statistical analysis

One-way ANOVA was applied for multiple comparisons, with Kruskal-Wallis post-test used to compare all groups and post comparison Dunn's or Mann Whitney test used to compare all other groups to the control group. All analyses were performed using GraphPad PRISM version 7.0 software. A P value < 0.05 was considered statistically significant.

3. Results

3.1. The Cry1Ac toxin provokes allergic manifestations

To define the allergenic potential of intra-gastric administration of Cry1Ac, we evaluated the allergic manifestations induced in sensitized mice, as specified in Fig. 1A, immediately after the intra-gastric challenge. In general, similar tolerance-related results were attained with both OVA doses tested, in the OVA alone groups, while in the OVA/CT groups with both OVA doses tested were attained the expected allergy-related responses although at higher magnitude with the higher OVA dose. For this reason, only the results with OVA 5 mg dose are shown for these groups in the Figs. 1–6. However, in the OVA/Cry1Ac groups in general with the higher OVA dose, the Cry1Ac-induced effects were lower than those elicited with the lower dose (50 μg). The results shown in the figures for OVA/Cry1Ac correspond to the OVA 5 mg dose unless other thing is specified.

The treatments with Cry1Ac, OVA/Cry1Ac and OVA/CT (with either of two OVA doses tested) induced mildly allergic manifestations, such as wheezing about mouth, nose and ears more than 10 times, pilar erecti and diarrhea (with score 3; Fig. 1B), while the PBS and OVA groups showed lower allergic manifestations (scores 1 and 2; Fig. 1B) in comparison with the rest of treatments. No significant differences in body temperature were detected between any of the groups (data not shown).

3.2. Cry1Ac treatment induces increased serum specific IgE response

Cry1Ac alone induced a significant increase of anti-Cry1Ac IgE in serum as compared with PBS group (Fig. 1C), while in SI and LI, no significant differences in the levels of anti-Cry1Ac IgE were observed between the treatments (Fig. 1D).

As expected, OVA/CT induced a significant increase of anti-OVA IgE levels in serum (Fig. 1E). However, in the SI only slight and similar anti-OVA IgE responses were detected in OVA/CT (Fig. 1F) and OVA/Cry1Ac treatments, however these responses were similar as those elicited in OVA group but higher with respect to PBS (Fig. 1F). The OVA/Cry1Ac and Cry1Ac groups were able to elicit systemic and mucosal Cry1Ac-specific IgG1, IgG2a and IgA antibody responses (Supplementary Fig. 2). Cry1Ac had a slight systemic adjuvant effect to OVA; however, the magnitude, is not comparable with the strong adjuvant effect of CT in this intestinal allergy model (Supplementary Fig. 3).

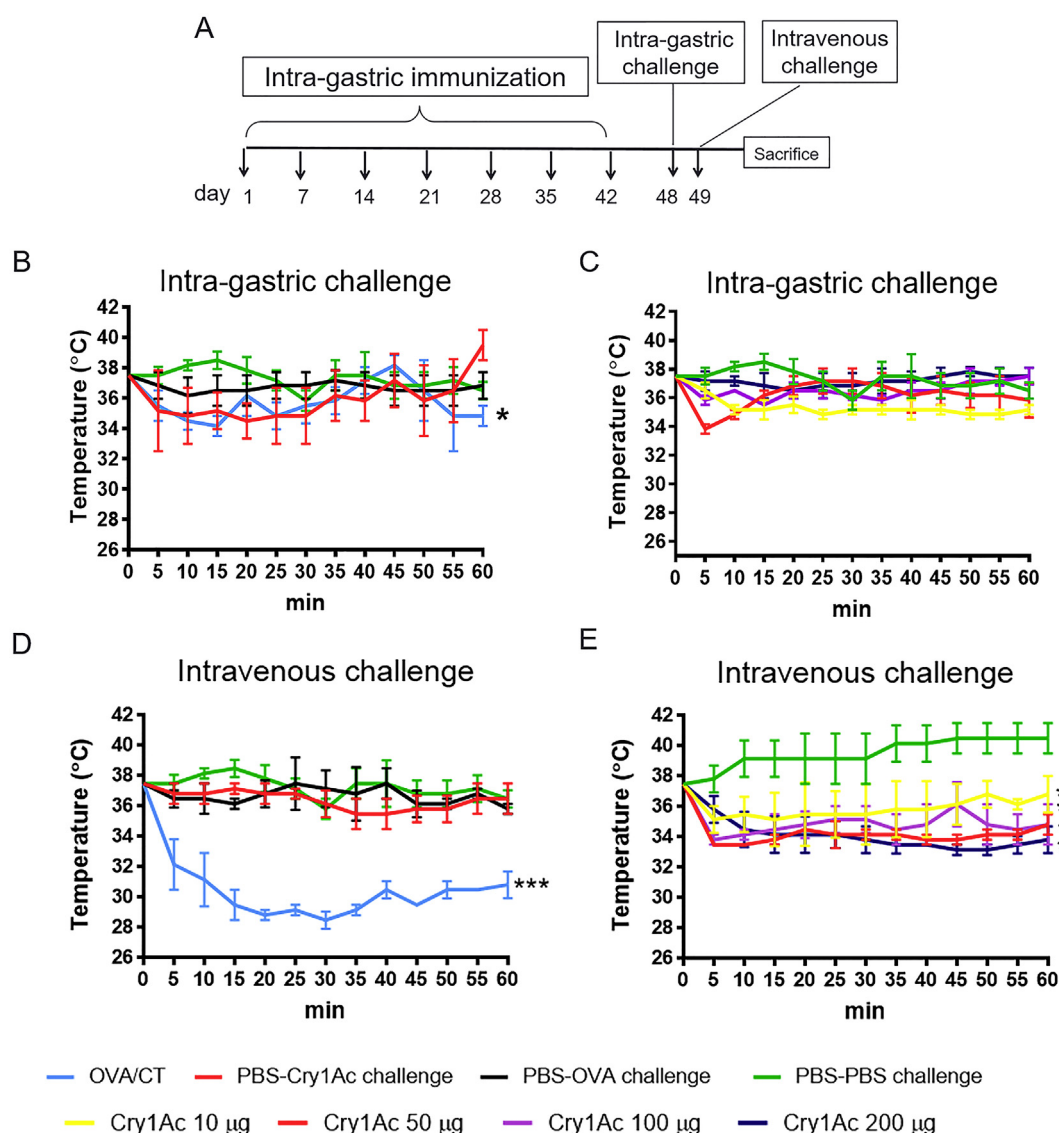


Fig. 6. Intravenous challenge with OVA or Cry1Ac results in anaphylaxis. (A) BALB/c mice were immunized once a week for seven weeks, on 48 day received an intra-gastrically and were assessing the rectal temperature for 1 h as specified in the section materials and methods and one day after (49) the mice received an intravenous antigen challenge with 100 µg of OVA for PBS groups and OVA/CT group, 100 µg of Cry1Ac for PBS group and the different Cry1Ac groups (10, 50, 100 or 200 µg of Cry1Ac). (B) the graph shows the mean of temperature measurement every 5 min after the intra-gastric challenge for 1 h of vehicle groups of PBS and OVA/CT allergy positive control, (C) mean of temperature measurement every 5 min h after the intra-gastric challenge of PBS group and (10, 50, 100 or 200 µg of Cry1Ac) groups, (D) shows the drop of the decrease in temperature assessed every 5 min for 1 h after intravenous challenge of PBS groups and OVA/CT allergy positive control and (E) the graph exhibited the drop of temperature after the intravenous challenge with 100 µg of Cry1Ac for every Cry1Ac group in comparison with the PBS vehicle group. The results shown in A-E were obtained using 3 to 5 mice per group. Significant differences ($P < 0.05$) are indicated as follows * versus PBS challenge (OVA); *** versus PBS challenge with (Cry1Ac, OVA, PBS) and * versus PBS challenge (PBS).

3.3. The intra-gastric administration of Cry1Ac induce a mixed cytokine profile

In general, a slightly increased mixed cytokine profile was detected in serum and in intestinal lavages of Cry1Ac, OVA/CT and OVA/Cry1Ac groups with respect to the PBS and OVA groups.

In serum the cytokine profile detected with significant increase were IL-10, IL-13, IL-6 and TNF α , (Fig. 2A) in Cry1Ac group, OVA/Cry1Ac and OVA/CT in comparison with PBS vehicle group. The cytokines were also evaluated in intestinal lavages, where a low mixed cytokine profile was observed in all groups, however only a slightly increased IL-6 level was detected in the OVA/CT and Cry1Ac groups (Fig. 2B). The difficulty in detecting intestinal cytokines, in this model of food allergy to OVA, may be due to the low levels induced or to their lability since in the OVA/CT group no substantial levels were detected either.

3.4. Cry1Ac treatment significantly increases the proportion of B220 + IgE + lymphocytes in spleen, mesenteric lymph node and Peyer's patches

The IgE antibody was also evaluated by flow cytometry, assessing the frequency of B220 + IgE + lymphocytes in cells from spleen, MLN, PP and lamina propria.

In spleen cells, Cry1Ac alone induced a considerable increase in the percentage of B220 + IgE + lymphocytes ($11.24\% \pm 3.72$) with respect to PBS ($0.82\% \pm 0.25$) and OVA ($1.23\% \pm 0.47$); while OVA/CT ($26.05\% \pm 3.24$) also exhibited an increase in this population ($P < 0.05$) (Fig. 3A, B). OVA/Cry1Ac also increased the proportion of B220 + IgE + lymphocytes but only with the 50 µg OVA dose ($54.7\% \pm 6.5$).

In MLN, Cry1Ac treatment elicited a significantly higher proportion of B220 + IgE + lymphocytes ($27.1\% \pm 8.57$) than was induced in PBS

Table 1

Histopathological changes recorded in the intestines of mice intra-gastrically administered. The histopathological changes recorded in sections of small intestine (SI) and large intestine (LI) of every group treated as described in materials and methods were marked as (+) positive or (–) negative when were present or absent in at least 70% of mice. Some observations are indicated in negative marked groups when histopathological changes were recorded in some individuals. The data summarize observations recorded in tissue sections from 9 mice per group.

	PBS	OVA	OVA/Cry1Ac	Cry1Ac	OVA/CT
SI					
Lymphoplasmacytic enteritis	(+) Mild, focal	(+) Mild, multifocal	(+) Mild, multifocal/diffuse	(+) Mild/moderate, diffuse/ multifocal	(+) Mild/moderate, diffuse
Goblet cell hyperplasia	(–)	(–)	(+) Mild/moderate/severe, diffuse	(+) Mild/moderate, multifocal	(+) Mild/moderate, diffuse
Lymphoid tissue hyperplasia	(–)	(–)	(+) Mild/moderate, focal	(+) Severe, focal	(+) Mild/moderate/severe, focal
Edema	(–)	(–)	(–)	(–)	(–)
Lymphangiectasia	(–)	(–)	(–)	(–)	(–)
Epithelial shedding	(–)	(–)	(–)	(–)	(–)
LI					
Lymphoplasmacytic enteritis	(–) Absent or mild, focal	(–) Absent or mild focal	(+) Mild, multifocal	(+) Mild, multifocal	(+) Mild, multifocal/diffuse
Goblet cell hyperplasia	(–) Absent or mild, focal	(–) Absent or Mild focal	(+) Mild/moderate multifocal	(+) Mild/moderate multifocal/ diffuse	(+) Mild/moderate diffuse
Lymphoid tissue hyperplasia	(–) Absent	(–) Absent or mild focal	(+) Mild/moderate, focal/ multifocal	(+) Mild/moderate, focal	(+) Mild/moderate to severe, focal
Edema	(–)	(–) Absent or mild diffuse	(+) Moderate, diffuse	(+) Mild, diffuse	(+) Moderate, diffuse
Lymphangiectasia	(–)	(–)	(–)	(+)	(+)
Epithelial shedding	(–)	(–)	(–)	(+)	(+)

(2.40% $\pm$ 1.45) and OVA (9.17% $\pm$ 4.10). OVA/Cry1Ac treatment also increased this population with the lower OVA dose 50 μ g (51.60% $\pm$ 20.1) but not with the high OVA dose 5 mg (1.33% $\pm$ 1.12). While OVA/CT (43.65% $\pm$ 12.65) also increased these population ($P < 0.05$) (Fig. 3A–B).

The percentage of B220+ IgE+ lymphocytes in PP was significantly increased in Cry1Ac (4.26% $\pm$ 1.46) in comparison to PBS (0.785% $\pm$ 0.21) and in OVA/Cry1Ac (39% $\pm$ 12.30) the increase was higher than every other group, but only with the low dose of OVA, while in OVA/CT was significantly increased (6.77% $\pm$ 3.72) with respect to PBS (0.78% $\pm$ 0.21) and OVA (2.13% $\pm$ 1.35) ($P < 0.05$) (Fig. 3A, B). In lamina propria cells of SI and LI, no significant changes in the cell population of B220+ IgE+ lymphocytes were detected between groups (data not shown).

3.5. The administration of Cry1Ac increases the number of eosinophils and total granulocytes in intestine

Eosinophils were analyzed by flow cytometry with Siglec-F mAb. In lamina propria cells of SI, the percentage of Siglec-F+ cells of OVA (3.68% $\pm$ 0.52), OVA/Cry1Ac (3.83% $\pm$ 0.52), Cry1Ac (6.27% $\pm$ 1.77), and OVA/CT (3.77% $\pm$ 0.44) treatments was significantly higher than in PBS (1.61% $\pm$ 0.42) groups (Fig. 4A–B). In the LI, OVA/CT (1.47% $\pm$ 0.25) showed a significant increase with respect to PBS (0.66% $\pm$ 0.10) in the percentage of Siglec-F+ cells, while a no significant increase was observed in the percentage of Siglec-F+ cells in OVA/Cry1Ac (1.01% $\pm$ 0.14) and Cry1Ac (2.15% $\pm$ 1.26) in LI ($P < 0.05$) (Fig. 4A, B).

The number of total granulocytes was also counted by microscopy in sections of SI and colon LI. The number of granulocytes per field was significantly increased in SI sections from OVA (6.56 $\pm$ 0.40), OVA/Cry1Ac (6.86 $\pm$ 0.46), Cry1Ac (9.08 $\pm$ 0.86) and OVA/CT (9.41 $\pm$ 0.44) compared to the PBS group (5.31 $\pm$ 0.35) ($P < 0.05$) (Fig. 4C). Likewise, in LI sections, the number of granulocytes per field were considerably increased in the OVA/Cry1Ac (6.76 $\pm$ 0.44) Cry1Ac (9.25 $\pm$ 0.68) and OVA/CT (9.41 $\pm$ 0.41) groups compared to the

PBS (2.85 $\pm$ 0.20) and OVA (4.16 $\pm$ 0.38) groups ($P < 0.05$) (Fig. 4D).

3.6. The Cry1Ac toxin does not induce an increase in the mMCP-1 levels in serum

It is well known that when IgE bound to its receptor (FcεRI) cross-link with allergens on mast cells, the mast cells suffer degranulation, releasing histamine, leukotrienes and proteases and provoking allergic symptoms. We measured the levels of mMCP-1, a mouse mast cell protease-1 and β -chymase because it is considered a typical allergic mediator released during mast cell degranulation [34] [35]. Only the OVA/CT (positive allergy control) treatment significantly increased the mMCP-1 levels in serum with respect to the rest of the groups (Fig. 4E). While OVA/Cry1Ac and Cry1Ac treatments showed mMCP-1 levels very similar to the PBS and OVA groups (Fig. 4E).

3.7. Intra-gastric administration of Cry1Ac induces lymphoid intestinal hyperplasia in colon

Histological analysis was performed in order to determine whether the intra-gastric administration of the different treatments induced pathological changes in tissues of intestine. In intestinal sections of control mice immunized with PBS and OVA the normal intestinal morphology was preserved. In contrast, in LI from mice treated with OVA/Cry1Ac, Cry1Ac or OVA/CT, intestinal lymphoid tissue hyperplasia was observed especially in the colon (Fig. 5). The cellular infiltrate in this hyperplasia appears to be formed by lymphocytes, and no inflammatory infiltrate of macrophages, eosinophils or neutrophils was observed inside in the H&E stained preparations. To determine whether the lymphoid hyperplasia was reversible once the immunizations were suspended, the intestinal histological analysis was done, in additional groups of mice which received the 8-week sensitization scheme, but were sacrificed until one month after. The histological analysis revealed that the presence intestinal hyperplasia persisted in the OVA/Cry1Ac, Cry1Ac or OVA/CT groups (data not shown).

In addition to the referred lymphoid hyperplasia in colon other

histopathological changes was identified in intestine, such as lymphoplasmacytic enteritis, goblet cell hyperplasia, edema, and epithelial shedding, were also recorded in particularly in the intestines (SI and LI) sections of OVA/CT, Cry1Ac and OVA/Cry1Ac groups (Table 1). In general, these alterations were more severe in the OVA/CT group than in the other groups.

3.8. The intravenous challenge with Cry1Ac provokes anaphylaxis

The mildly allergenic responses recorded in the groups sensitized with Cry1Ac by intra-gastric route led us to prove whether this toxin may provoke anaphylaxis if was administered at higher doses (Fig. 6C). After intra-gastric challenge only in the OVA/CT group was recorded a slight temperature drop (Fig. 6B). However, a limitation of the oral sensitization food allergy model selected, is that symptoms of anaphylaxis are not achieved upon food gavage [31], so we performed intravenous antigen challenge with OVA or with Cry1Ac in additional groups (Fig. 6A). Remarkably, in mice intragastrically sensitized with every dose tested of Cry1Ac (10, 50, 100 or 200 µg) a significant temperature drop was recorded after the intravenous challenge with Cry1Ac (Fig. 6E). This drop was observed since the first minutes and maintained during 35 min, while no significant temperature drops were recorded after intra-gastric challenges with Cry1Ac (Fig. 6C). While a marked temperature drop was observed after intravenous challenge with OVA in OVA/CT allergy positive control (Fig. 6D). This drop was more pronounced than the one elicited with Cry1Ac further reinforcing the higher allergenic potential of CT. As expected, nor intra-gastric neither intravenous challenges with OVA, Cry1Ac or PBS provoked significant changes in temperature in PBS group (Fig. 6B,D).

3.9. The binding OVA to macrophage's cell surface is competed by Cry1Ac

A possible explanation to the lower effect Cry1Ac induced with the 5 mg OVA dose, with respect to the 50 µg dose in the OVA/Cry1Ac treatments, which was recorded for some allergy parameters (i.e. increase in B220+ IgE+ and IgE+ Siglec-F+ cells) could be that OVA and Cry1Ac were competing with a common receptor. The preliminary results performed in macrophages support this possibility as the binding of OVA-FITC to the cell surface was partially inhibited by an excess of Cry1Ac (Supplementary Fig. 4). Considering that OVA is known to be endocytosed in macrophages through the mannose receptor further studies are still required to determine whether Cry1Ac use this same receptor. It will also be important to determine if OVA could block some of the Cry1Ac-induced activation effects on macrophages [24,36].

4. Discussion

Overall, the results indicate that Cry1Ac toxin via intra-gastric route has the capability to generate moderate allergy-related immune responses. As, we found the groups administered with Cry1Ac and OVA/Cry1Ac, but particularly the Cry1Ac alone group, elicited some allergy-related manifestations, that were comparable in magnitude to those induced by the allergy positive control (OVA/CT treatment). For example: moderate score (level 3) of systemic allergic reactions, increased proportion IgE+ cells in spleen, MLN and PP, increased proportion of granulocytes in the intestinal lamina propria and intestinal tissue, and generation of colonic lymphoid hyperplasia. However, the mucosal adjuvant effect of CT, measured by the increase on the specific antibody responses towards co-administered antigen OVA, was much greater than the slight adjuvant effect of Cry1Ac elicited by intra-gastric route. Also, the increases in Mmcp-1 levels and magnitude of temperature drop following intravenous challenge were more pronounced in the OVA/CT than in the Cry1Ac groups indicating a lower potential of Cry1Ac to promote allergy and anaphylaxis with respect to CT.

The mildly allergic manifestations elicited in present study by treatments with Cry1Ac and OVA/CT were similar to those recorded in

the comparable murine model of food allergy to OVA established by Perrier et al. [26] Likewise, mildly allergy manifestations such as cramps and scarring of skin are observed in patients with food allergy [37].

Our results indicate that Cry1Ac toxin is immunogenic when administered by intra-gastric route as OVA/Cry1Ac and Cry1Ac treatments induced systemic and mucosal antibody responses (Supplementary Fig. 3). Likewise, we have previously reported the immunogenicity of Cry1Ac, Cry1Ab, Cry1Aa and Cry3A toxins but following administration by intranasal and intraperitoneal routes, using a scheme consisting in 3 weekly immunizations [19]. The adjuvant capacity of Cry1Ac by intra-gastric route is slight, as by the coadministration of OVA with Cry1Ac only were induced significant OVA-specific IgG1 and IgA antibody responses in serum (Supplementary Fig. 4); and these responses were much lower than those induced in the OVA/CT group, thus as expected CT clearly exhibited a potent systemic and mucosal adjuvant effect similar to the reported in other studies [26,27,38].

By flow cytometry and microscopy, we found that the numbers of eosinophils were significantly increased in the gut of the positive allergy control OVA/CT, as well as in OVA/Cry1Ac and Cry1Ac groups. Similar to our results, the increase in the number of eosinophils numbers has been observed in the blood in several food allergy models using adjuvants [39]. Also in a study of Zeljenková et al. [9] assessing the effect of feeding rats with 11% or 33% GM maize that express Cry1Ab toxin (MON810) for 5 and 14 weeks were found increased numbers of eosinophils in blood, but they did not perform any examination of allergy related parameters at the intestines. In addition, they found that rats fed GM maize presented some signs of hepatorenal toxicity; however, they concluded that MON810 was as safe as conventional varieties of corn.

In most studies in which the biosafety of GM Bt plants has been tested, the possible changes or damages that Cry toxins could provoke in organs such as the intestine are not microscopically evaluated. For this reason, we performed histological analysis of intestinal tissue. The main change observed in OVA/Cry1Ac, Cry1Ac and OVA/CT treated groups was a lymphoid intestinal hyperplasia recorded in LI sections. The presence of colon lymphoid hyperplasia is a parameter that had not been described associated with food allergy in murine models, according to present results we propose that it should be considered as it.

In humans, isolated lymphoid follicles are normally found throughout the intestines but their presence is known to be increased in the caecum and rectum regions. However, a high density of lymphoid follicles might imply a pathological condition. Besides, under inflammatory conditions, the number, diameter and density of isolated lymphoid follicles is increased [40]. Some studies have detected nodular lymphoid intestinal hyperplasia associated with *Giardia* infection, *H. pylori* infection and with immune deficiency status such as IgA deficiency. In most of these cases, the lymphoid hyperplasia was observed in SI [41,42]. Whereas, coinciding with our findings, intestinal lymphoid hyperplasia of the colon is more commonly related to food allergy in children and adults [43,44]. Likewise, some studies have consistently demonstrated an association between food allergy and lymphoid hyperplasia in colon, because the number of patients without colonic lymphoid hyperplasia that had food allergy was much lower than those presenting it [45,46].

In addition to its association with food allergy, the presence of nodular lymphoid hyperplasia (NLH) has been also linked with inflammatory bowel disease (IBD), celiac disease (CeD), lymphoma, colonic carcinoma [47–51] and has been proposed as a marker of low-grade inflammation in inflammatory bowel disease. In children NLH are considered parapsychological aggregates, as they are regularly asymptomatic and reversible. Conversely, although little is known about the clinical implications of NLH in adults, considering the risk of malignant transformation the endoscopic surveillance of these structures has been recommended [42]. On basis of present results showing lymphoid

colonic hyperplasia induced by intra-gastric administration of Cry1Ac, we consider important to perform further studies to determine whether the ingestion of Cry toxins maybe associated with an increased presence of nodular lymphoid hyperplasia in humans, as these structures appear to be related with food allergy and/or with intestinal inflammatory conditions.

Our present results demonstrating the potential of Cry1Ac to induce responses linked with food allergy differ from the effects reported with Cry1Ab toxin using an experimental allergic asthma or disease relapse model induced with OVA, as they did not observe allergic manifestations. In the study of Reiner et al. [52] mice consumed approximately 19 µg per day of Cry1Ab toxin, as they were feed with a diet including 33% Bt maize containing approximately 0.0013% of Cry1Ab during 32 or 34 days. The different results may due to the distinct Cry1A protein tested, by the distinct schedule, different allergy model used and distinct dose. However, the authors argued that the absence of allergic response could be due to the denaturation of Cry1Ab during food processing.

Likewise, in a recent study of Andreassen et al. [25] was found no evidence of Cry1Ab toxin immunogenicity or allergenicity to the allergen lupin in mice, after repeated intra-gastric administrations of low doses of Cry1Ab (10 µg) and intraperitoneal challenges. However, in their positive allergy group coadministered with CT neither were detected significant specific antibody responses, nor changes in serum cytokines. These different results could also be explained by the differences in sequence between Cry1Ab and Cry1Ac, or to the different doses and routes of administration used, as well as by the differences derived from the modification of native cry gene sequences done to increase the effectivity in a GM Bt plants [53,54]. So further detailed studies are required in which the immunogenicity and allergenicity of these similar Cry1A proteins are compared in appropriate conditions to determine this apparent distinct allergenic potential.

Despite our results contrast to other studies, finding no evidence of immunogenicity nor allergenicity for Cry1Ab, those were performed without considering the evaluation of allergy-related intestinal parameters or using low doses of antigen or a reduced number of immunizations. Thus the appropriate use of antigen doses, immunization schedule, food-allergy model and the analysis intestinal parameters appear to be critical factors to precisely evaluate the immunogenicity and allergenicity of Cry proteins via intra-gastric route. With the dose of 50 µg protein/25 g mouse we were able to observe the immunogenicity and moderate allergenic potential of Cry1Ac in a food allergy mouse model, extrapolating these data to a 70 kg person, 140 mg should be the dose required to induce immunogenic effects. These levels could not be easily achieved by consumption of some GM plants in which the expression levels of Cry proteins had been estimated to be very low (i.e. 0.0013% of protein content in MON810 maize [52]), however there are wide variations in measurements of Cry concentrations in plant material [55] and in other transgenic plants such as broccoli, much higher expression levels of Cry proteins had been achieved (i.e. 0.4% of total protein). Besides, it is important to mention that the expression levels of Cry proteins in plants can differ considerably [55]. Considering these aspects, the dose required to observe an immunostimulating or allergenic effect due to Cry1Ac could be achieved by ingestion of a much lower amount of GM plant than was originally thought.

It is important to note that in this study we used a native purified Cry1Ac toxin, while the proteins expressed in transgenic plants are modified or truncated, so as a result of these modifications they may have different immunological properties. We also recommend, the need to perform such further evaluation of the allergenicity of each transgenic plant using appropriate food allergy models like the one used in this work, as well as to accomplish an integral evaluation of the intestinal immune response.

5. Conclusion

Cry1Ac has the potential capability to provoke, related allergy changes when administered by intra-gastric route. In addition, present study contributes to propose additional parameters that had not been considered in food allergy models such as the presence of colonic lymphoid hyperplasia, assessing the lymph nodes and Peyer's patches and of IgE+ SiglecF+ cells intestinal lamina propria; as they were exclusively detected elevated in the allergy positive group (OVA/CT) and in the Cry1Ac groups.

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Conflict of interest

The authors declare no conflicts of interest or financial conflicts.

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References

- [1] A. Bravo, S. Jansens, M. Peferoen, Immunocytochemical localization of *Bacillus thuringiensis* insecticidal crystal proteins in intoxicated insects, *J. Invertebr. Pathol.* 60 (1992) 237–246, [http://dx.doi.org/10.1016/0022-2011\(92\)90004-N](http://dx.doi.org/10.1016/0022-2011(92)90004-N).
- [2] X. Zhang, M. Candas, N.B. Griko, R. Taussig, L.A. Bulla, A mechanism of cell death involving an adenyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 9897–9902, <http://dx.doi.org/10.1073/pnas.0604017103>.
- [3] F.S. Betz, B.G. Hammond, R.L. Fuchs, Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests, *Regul. Toxicol. Pharmacol.* 32 (2000) 156–173, <http://dx.doi.org/10.1006/rtp.2000.1426>.
- [4] K. Adel-Patient, V.D. Guimaraes, A. Paris, M.-F. Drumare, S. Ah-Leung, P. Lamourette, M.-C. Nevers, C. Canlet, J. Molina, H. Bernard, C. Crémion, J.-M. Wal, Immunological and metabolic impacts of administration of Cry1Ab protein and MON 810 maize in mouse, *PLoS One* 6 (2011) e16346, <http://dx.doi.org/10.1371/journal.pone.0016346>.
- [5] A. Finamore, M. Roselli, S. Britti, G. Monastera, R. Ambra, A. Turrini, E. Mengheri, Intestinal and peripheral immune response to MON810 maize ingestion in weaning and old mice, *J. Agric. Food Chem.* 56 (2008) 11533–11539, <http://dx.doi.org/10.1021/jf802059w>.
- [6] V.D. Guimaraes, M.-F. Drumare, S. Ah-Leung, D. Lereclus, H. Bernard, C. Crémion, J.-M. Wal, K. Adel-Patient, Cry1Ab protein and cholera toxin on allergic sensitisation and elicitation to peanut, *Food Agric. Immunol.* 19 (2008) 325–337, <http://dx.doi.org/10.1080/09540100802495651>.
- [7] B. Koul, R. Yadav, I. Sanyal, D.V. Amla, Comparative performance of modified full-length and truncated *Bacillus thuringiensis*-cry1Ac genes in transgenic tomato, *Springerplus* 4 (2015) 203, <http://dx.doi.org/10.1186/s40064-015-0991-x>.
- [8] P. Liu, X. He, D. Chen, Y. Luo, S. Cao, H. Song, T. Liu, K. Huang, W. Xu, A 90-day subchronic feeding study of genetically modified maize expressing Cry1Ac-M protein in Sprague-Dawley rats, *Food Chem. Toxicol.* 50 (2012) 3215–3221, <http://dx.doi.org/10.1016/j.fct.2012.06.009>.
- [9] D. Zeljenková, K. Ambušová, M. Bartušová, A. Kebis, J. Kovřížnych, Z. Krivošíková, M. Kuricová, A. Lišková, E. Rollerová, V. Spustová, E. Szabová, J. Tulinská, S. Wimmerová, M. Levkut, V. Révájová, Z. Ševčíková, K. Schmidt, J. Schmidtk, J.L. La Paz, M. Corujo, M. Pla, G.A. Kleter, E.J. Kok, J. Sharbati, C. Hanisch, R. Einspanier, K. Adel-Patient, J.-M. Wal, A. Spök, A. Pötting, C. Kohl, R. Wilhelm, J. Schiemann, P. Steinberg, Ninety-day oral toxicity studies on two genetically modified maize MON810 varieties in Wistar Han RCC rats (EU 7th Framework Programme project GRACE), *Arch. Toxicol.* 88 (2014) 2289–2314, <http://dx.doi.org/10.1007/s00204-014-1374-8>.
- [10] M. Zhang, Q. Zhuo, Y. Tian, J. Piao, X. Yang, Long-term toxicity study on transgenic rice with Cry1Ac and sck genes, *Food Chem. Toxicol.* 63 (2014) 76–83, <http://dx.doi.org/10.1016/j.fct.2013.10.035>.
- [11] D.S. Kotlyar, M. Shum, J. Hsieh, W. Blonski, D.A. Greenwald, Non-pulmonary allergic diseases and inflammatory bowel disease: a qualitative review, *World J. Gastroenterol.* 20 (2014) 11023–11032, <http://dx.doi.org/10.3748/wjg.v20.i32>.

- 11023.
- [12] L. Moreno-Fierros, N. García, R. Gutiérrez, R. López-Revilla, R.I. Vázquez-Padrón, Intranasal, rectal and intraperitoneal immunization with protoxin Cry1Ac from *Bacillus thuringiensis* induces compartmentalized serum, intestinal, vaginal and pulmonary immune responses in Balb/c mice, *Microbes Infect.* 2 (2000) 885–890, [http://dx.doi.org/10.1016/S1286-4579\(00\)00398-1](http://dx.doi.org/10.1016/S1286-4579(00)00398-1).
 - [13] R.I. Vázquez, L. Moreno-Fierros, L. Neri-Bazán, G.A. De La Riva, R. López-Revilla, *Bacillus thuringiensis* Cry1Ac protoxin is a potent systemic and mucosal adjuvant, *Scand. J. Immunol.* 49 (1999) 578–584, <http://dx.doi.org/10.1046/j.1365-3083.1999.00534.x>.
 - [14] M. Legorreta-Herrera, R. Oviedo Meza, L. Moreno-Fierros, Pretreatment with Cry1Ac protoxin modulates the immune response, and increases the survival of plasmodium-infected CBA/Ca mice, *J Biomed Biotechnol* 2010 (2010), <http://dx.doi.org/10.1155/2010/198921>.
 - [15] A. Jarillo-Luna, L. Moreno-Fierros, R. Campos-Rodríguez, M.A. Rodríguez-Monroy, E. Lara-Padilla, S. Rojas-Hernández, Intranasal immunization with *Naegleria fowleri* lysates and Cry1Ac induces metaplasia in the olfactory epithelium and increases IgA secretion, *Parasite Immunol.* 30 (2008) 31–38, <http://dx.doi.org/10.1111/j.1365-3024.2007.00999.x>.
 - [16] E. González-González, A.L. García-Hernández, R. Flores-Mejía, R. López-Santiago, L. Moreno-Fierros, The protoxin Cry1Ac of *Bacillus thuringiensis* improves the protection conferred by intranasal immunization with *Brucella abortus* RB51 in a mouse model, *Vet. Microbiol.* 175 (2015) 382–388, <http://dx.doi.org/10.1016/j.vetmic.2014.11.021>.
 - [17] S. Ibarra-Moreno, A.L. García-Hernández, L. Moreno-Fierros, Coadministration of protoxin Cry1Ac from *Bacillus thuringiensis* with metacystode extract confers protective immunity to murine cysticercosis, *Parasite Immunol.* 36 (2014) 266–270, <http://dx.doi.org/10.1111/pim.12103>.
 - [18] G.G. Guerrero, L. Moreno-Fierros, Carrier potential properties of *Bacillus thuringiensis* Cry1A toxins for a diphtheria toxin epitope, *Scand. J. Immunol.* 66 (2007) 610–618, <http://dx.doi.org/10.1111/j.1365-3083.2007.01992.x>.
 - [19] G.G. Guerrero, D.H. Dean, L. Moreno-Fierros, Structural implication of the induced immune response by *Bacillus thuringiensis* cry proteins: role of the N-terminal region, *Mol. Immunol.* 41 (2004) 1177–1183, <http://dx.doi.org/10.1016/j.molimm.2004.06.026>.
 - [20] L. Moreno-Fierros, A.L. García-Hernández, D. Ilhuicatzí-Alvarado, L. Rivera-Santiago, M. Torres-Martínez, N. Rubio-Infante, M. Legorreta-Herrera, Cry1Ac protoxin from *Bacillus thuringiensis* promotes macrophage activation by upregulating CD80 and CD86 and by inducing IL-6, MCP-1 and TNF- α cytokines, *Int. Immunopharmacol.* 17 (2013) 1051–1066, <http://dx.doi.org/10.1016/j.intimp.2013.10.005>.
 - [21] N. Rubio-Infante, D. Ilhuicatzí-Alvarado, M. Torres-Martínez, J.P. Reyes-Grajeda, R. Nava-Acosta, E. González-González, L. Moreno-Fierros, The macrophage activation induced by *Bacillus thuringiensis* Cry1Ac protoxin involves ERK1/2 and p38 pathways and the interaction with cell-surface-HSP70, *J. Cell. Biochem.* 119 (2018) 580–598, <http://dx.doi.org/10.1002/jcb.26216>.
 - [22] N. Rubio-Infante, L. Moreno-Fierros, An overview of the safety and biological effects of *Bacillus thuringiensis* Cry toxins in mammals, *J. Appl. Toxicol.* (2015), <http://dx.doi.org/10.1002/jat.3252> (n/a–n/a).
 - [23] L. Moreno-Fierros, A.L. García-Hernández, D. Ilhuicatzí-Alvarado, L. Rivera-Santiago, M. Torres-Martínez, N. Rubio-Infante, M. Legorreta-Herrera, Cry1Ac protoxin from *Bacillus thuringiensis* promotes macrophage activation by upregulating CD80 and CD86 and by inducing IL-6, MCP-1 and TNF- α cytokines, *Int. Immunopharmacol.* 17 (2013) 1051–1066, <http://dx.doi.org/10.1016/j.intimp.2013.10.005>.
 - [24] M. Torres-Martínez, N. Rubio-Infante, A.L. García-Hernández, R. Nava-Acosta, D. Ilhuicatzí-Alvarado, L. Moreno-Fierros, Cry1Ac toxin induces macrophage activation via ERK1/2, JNK and p38 mitogen-activated protein kinases, *Int. J. Biochem. Cell Biol.* 78 (2016) 106–115, <http://dx.doi.org/10.1016/j.biocel.2016.06.022>.
 - [25] M. Andreassen, T. Bohn, O.-G. Wikmark, J. Bodin, T. Traavik, M. Lovik, U.C. Nygaard, Investigations of immunogenic, allergenic and adjuvant properties of Cry1Ab protein after intragastric exposure in a food allergy model in mice, *BMC Immunol.* 17 (2016) 10, <http://dx.doi.org/10.1186/s12865-016-0148-x>.
 - [26] C. Perrier, A.C. Thierry, A. Mercenier, B. Corthésy, Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice, *Clin. Exp. Allergy* 40 (2010) 153–162, <http://dx.doi.org/10.1111/j.1365-2222.2009.03329.x>.
 - [27] K. Ganeshan, C.V. Neilsen, A. Hadsaitong, R.P. Schleimer, X. Luo, P.J. Bryce, Impairing oral tolerance promotes allergy and anaphylaxis: a new murine food allergy model, *J. Allergy Clin. Immunol.* 123 (2009), <http://dx.doi.org/10.1016/j.jaci.2008.10.011> (231–238.e4).
 - [28] M. Chehade, L. Mayer, Oral tolerance and its relation to food hypersensitivities, *J. Allergy Clin. Immunol.* 115 (2005) 3–12, <http://dx.doi.org/10.1016/j.jaci.2004.11.008> (quiz 13).
 - [29] S. Rojas-Hernández, M.A. Rodríguez-Monroy, R. López-Revilla, A.A. Reséndiz-Albor, L. Moreno-Fierros, Intranasal coadministration of the Cry1Ac protoxin with amoebal lysates increases protection against *Naegleria fowleri* meningoencephalitis, *Infect. Immun.* 72 (2004) 4368–4375, <http://dx.doi.org/10.1128/IAI.72.8.4368-4375.2004>.
 - [30] J.L. Van Gramberg, M.J. de Veer, R.E. O'Hehir, E.N.T. Meeusen, R.J. Bischof, Use of animal models to investigate major allergens associated with food allergy, *J Allergy Ther* 2013 (2013) 635695, <http://dx.doi.org/10.1155/2013/635695>.
 - [31] L.M. Bartnikas, M.F. Gurish, O.T. Burton, S. Leisten, E. Janssen, H.C. Oettgen, J. Beaupré, C.N. Lewis, K.F. Austen, S. Schulte, J.L. Hornick, R.S. Geha, M.K. Oyoshi, Epicutaneous sensitization results in IgE-dependent intestinal mast cell expansion and food-induced anaphylaxis, *J. Allergy Clin. Immunol.* 131 (2013), <http://dx.doi.org/10.1016/j.jaci.2012.11.032> (451–60.e1–6).
 - [32] S.L. Verdin-Terán, A. Vilches-Flores, L. Moreno-Fierros, Immunization with Cry1Ac from *Bacillus thuringiensis* increases intestinal IgG response and induces the expression of FcRn in the intestinal epithelium of adult mice, *Scand. J. Immunol.* 70 (2009) 596–607, <http://dx.doi.org/10.1111/j.1365-3083.2009.02332.x>.
 - [33] A.A. Reséndiz-Albor, R. Esquivel, R. López-Revilla, L. Verdín, L. Moreno-Fierros, Striking phenotypic and functional differences in lamina propria lymphocytes from the large and small intestine of mice, *Life Sci.* 76 (2005) 2783–2803, <http://dx.doi.org/10.1016/j.lfs.2004.08.042>.
 - [34] S.J. Galli, M. Tsai, A.M. Piliponsky, The development of allergic inflammation, *Nature* 454 (2008) 445–454, <http://dx.doi.org/10.1038/nature07204>.
 - [35] M.-Y. Chung, H.S. Shin, D.W. Choi, D.-H. Shon, Citrus Tachibana leaf extract mitigates symptoms of food allergy by inhibiting Th2-associated responses, *J. Food Sci.* (2016), <http://dx.doi.org/10.1111/1750-3841.13315>.
 - [36] D.J. Viguerst, S. Vick, V.L. Shepherd, Characterization of functional mannose receptor in a continuous hybridoma cell line, *BMC Immunol.* 13 (2012) 51, <http://dx.doi.org/10.1186/1471-2172-13-51>.
 - [37] H.A. Sampson, Update on food allergy, *J. Allergy Clin. Immunol.* 113 (2004) 805–819, <http://dx.doi.org/10.1016/j.jaci.2004.03.014>.
 - [38] C.B. Mathias, S.A. Hobson, M. García-Lloret, G. Lawson, D. Poddighe, E.J. Freyschmidt, W. Xing, M.F. Gurish, T.A. Chatila, H.C. Oettgen, IgE-mediated systemic anaphylaxis and impaired tolerance to food antigens in mice with enhanced IL-4 receptor signaling, *J. Allergy Clin. Immunol.* 127 (2011) 795–805, <http://dx.doi.org/10.1016/j.jaci.2010.11.009>.
 - [39] E.B. Brandt, R.T. Strait, D. Hershko, Q. Wang, E.E. Muntel, T.A. Scribner, N. Zimmermann, F.D. Finkelmann, M.E. Rothenberg, Mast cells are required for experimental oral allergen – induced diarrhea, *J. Clin. Invest.* 112 (2003), <http://dx.doi.org/10.1172/JCI200319785.Introduction>.
 - [40] F. Sipos, G. Muzes, Isolated lymphoid follicles in colon: switch points between inflammation and colorectal cancer? *World J. Gastroenterol.* 17 (2011) 1666–1673, <http://dx.doi.org/10.3748/wjg.v17.i13.1666>.
 - [41] P. Mansueto, G. Iacono, A. Seidita, A. D'Alcamo, D. Sprini, A. Carroccio, Review article: intestinal lymphoid nodular hyperplasia in children — the relationship to food hypersensitivity, *Aliment. Pharmacol. Ther.* (2012) 1000–1009, <http://dx.doi.org/10.1111/j.1365-2036.2012.05062.x>.
 - [42] A. Albuquerque, Nodular lymphoid hyperplasia in the gastrointestinal tract in adult patients: a review, *World J. Gastrointest. Endosc.* 6 (2014) 534, <http://dx.doi.org/10.4253/wjge.v6.i11.534>.
 - [43] M. Kojima, N. Nakamura, H. Itoh, T. Motoori, K. Hoshi, Y. Enomoto, T. Johshita, H. Nakamine, Histological variety of localized lymphoid hyperplasia of the large intestine: histopathological, immunohistochemical and genotypic findings of 16 cases, *J. Clin. Exp. Hematop.* 49 (2009) 15–21, <http://dx.doi.org/10.3960/jslrt.49.15>.
 - [44] R. Troncone, V. Discepolo, Colon in food allergy, *J. Pediatr. Gastroenterol. Nutr.* 48 (Suppl 2) (2009) S89–S91, <http://dx.doi.org/10.1097/MPG.0b013e3181a15d1a>.
 - [45] J. Kokkonen, M. Haapalahti, K. Laurila, T.J. Karttunen, M. Mäki, Cow's milk protein-sensitive enteropathy at school age, *J. Pediatr.* 139 (2001) 797–803, <http://dx.doi.org/10.1067/mpd.2001.118882>.
 - [46] G. Iacono, A. Ravelli, L. Di Prima, C. Scalici, S. Bolognini, S. Chiappa, G. Pirrone, G. Licastri, A. Carroccio, Colonic lymphoid nodular hyperplasia in children: relationship to food hypersensitivity, *Clin. Gastroenterol. Hepatol.* 5 (2007) 361–366, <http://dx.doi.org/10.1016/j.cgh.2006.12.010>.
 - [47] R.A. Bronen, S.N. Glick, S.K. Teplick, Diffuse lymphoid follicles of the colon associated with colonic carcinoma, *Am. J. Roentgenol.* 142 (1984) 105–109, <http://dx.doi.org/10.2214/ajr.142.1.105>.
 - [48] R. Nascimbeni, F. Di Fabio, E. Di Betta, P. Mariani, S. Fisogni, V. Villanacci, Morphology of colorectal lymphoid aggregates in cancer, diverticular and inflammatory bowel disease, *Mod. Pathol.* 18 (2005) 681–685, <http://dx.doi.org/10.1038/modpathol.3800343>.
 - [49] O. Bernardi, P.M. Dourado, R.A. Carvalho, S. Martinelli, G.U. Berger, G.P. Head, C. Omoto, High levels of biological activity of Cry1Ac protein expressed on MON 87701 $\times$ MON 89788 soybean against *Heliothis virescens* (Lepidoptera: Noctuidae), *Pest Manag. Sci.* 70 (2014) 588–594, <http://dx.doi.org/10.1002/ps.3581>.
 - [50] P.J. Kenney, R.E. Koehler, G.D. Shackelford, The clinical significance of large lymphoid follicles of the colon, *Radiology* 142 (1982) 41–46, <http://dx.doi.org/10.1148/radiology.142.1.7053546>.
 - [51] A.C. Piscaglia, L. Laterza, V. Cesario, V. Gerardi, R. Landi, L.R. Lopetuso, G. Galò, G. Fabbretti, M. Brisigotti, M.L. Stefanelli, A. Gasbarrini, Nodular lymphoid hyperplasia: a marker of low-grade inflammation in irritable bowel syndrome? *World J. Gastroenterol.* 22 (2016) 10198–10209, <http://dx.doi.org/10.3748/wjg.v22.i46.10198>.
 - [52] D. Reiner, R.-Y. Lee, G. Dekan, M.M. Epstein, No adjuvant effect of bacillus thuringiensis-maize on allergic responses in mice, *PLoS One* 9 (2014) e103979, <http://dx.doi.org/10.1371/journal.pone.0103979>.
 - [53] H. Li, B. Oppert, K.Y. Zhu, R.A. Higgins, F. Huang, L.L. Buschman, Transgenic plants expressing Bt delta-endotoxins, *Entomol. Sin.* (2003) 1.
 - [54] Y. Devos, A. De Schrijver, P. De Clercq, J. Kiss, J. Romeis, Bt-maize event MON 88017 expressing Cry3Bb1 does not cause harm to non-target organisms, *Transgenic Res.* 21 (2012) 1191–1214, <http://dx.doi.org/10.1007/s11248-012-9617-z>.
 - [55] I. Ishaaya, S.R. Palli, A.R. Horowitz, *Advanced Technologies for Managing Insect Pests*, Springer, 2012.

Supplementary Fig. 1.

Standardization of positive allergy control in an allergy food model used to OVA doses. Utilized the allergy food model as described in materials and methods by intra-gastric route assessing a two doses of OVA (50 µg and 5 mg) plus CT 10 µg, spleen and MLN cells were obtained stained with mAbs and analyzed by flow cytometry for assessing positive allergy parameters as Gr1+ cells (polymorphonuclear cells) and Gr1+ IgE+ cells. (A) Shown representative dot plots with the percentage of Gr1+ cells for every group, (B) percentage of Gr1+ IgE+ cells, (C) the graphics shows mean of the cytokine levels Th1/Th2 quantified by ELISA in serum, (D) mean of every group that shows the optic density (OD 405 nm) of OVA-specific antibody levels of IgG1, IgG2a and IgE in serum. The graphs show the mean values ± SEM from each group (n = 5 mice per group). Significant differences ($P < 0.05$) are indicated as follows: * versus PBS.

Supplementary Fig. 2.

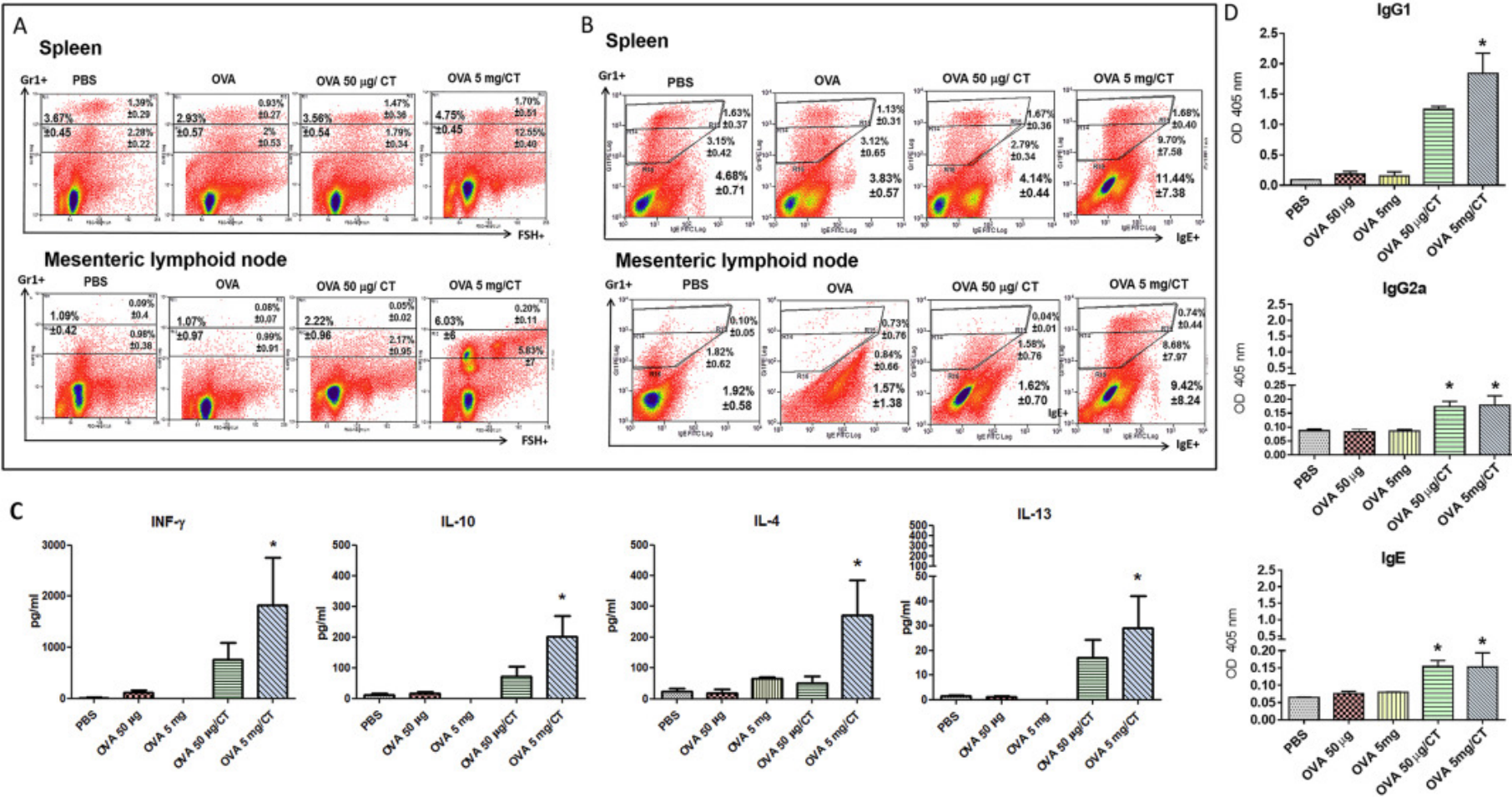
Cry1Ac toxin is immunogenic when was administered intra-gastrically with a preferentially Cry1Ac-specific IgG1 antibody response in serum. The Cry1Ac-specific IgG1, IgG2a and IgA antibody response were determined by ELISA in serum samples and intestinal lavages of small intestine (SI) and large intestine (LI) obtained from BALB/c mice immunized as described in materials and methods. Bar graphs show the levels of Cry1Ac-specific antibodies in (A) serum, (B) SI (open bars) and LI (filled bars). The data presented in bar plots are expressed as antibody titers for IgG1, IgG2a and IgA in serum and the optical density (OD 405 nm) for IgG1, IgG2a and IgA in intestine. Graphs show mean ± SEM values, of data obtained from independent experiments using 4 mice per group (n = 8 to 12 mice per group) and the significant differences ($P < 0.05$) are indicated as * versus PBS and * versus PBS challenge (PBS).

Supplementary Fig. 3.

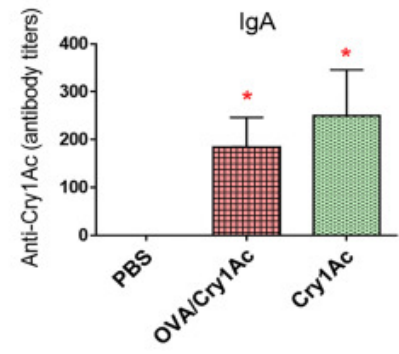
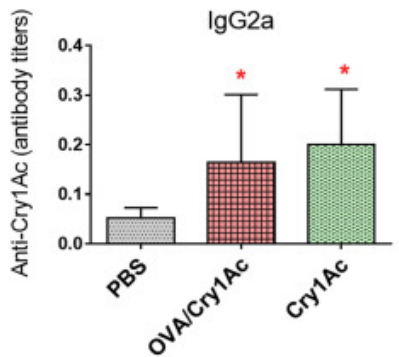
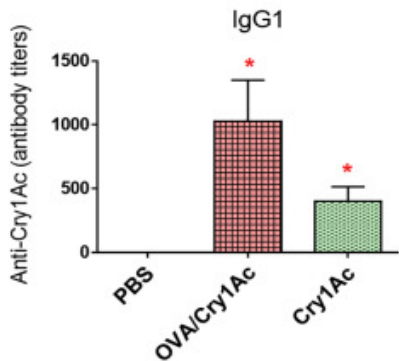
Cry1Ac toxin has a slight adjuvant effect to OVA on IgG1 in serum. The OVA-specific antibody response was determined by ELISA. (A) Response of OVA-specific antibodies in serum; the bar plots show the mean of antibody titers for IgG1, IgG2a and IgA, (B) response of OVA-specific antibodies in intestinal lavages of SI (open bars) and large LI (filled bars); the bar plots show the mean of (OD 405 nm) for IgG1, IgG2a and IgA. The graphs show the mean values ± SEM of data obtained from independent experiments using 4 mice per group (n = 8 to 12 mice per group); significant differences ($P < 0.05$) are indicating as follows: * versus PBS and & versus OVA.

Supplementary Fig. 4.

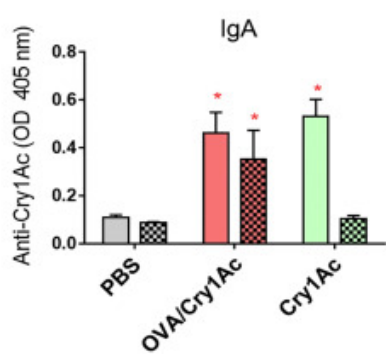
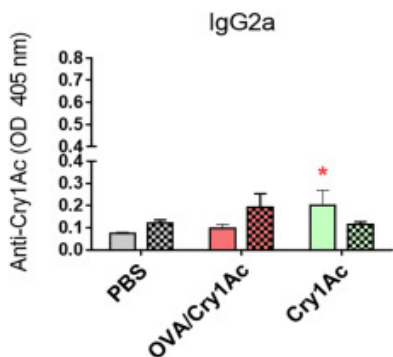
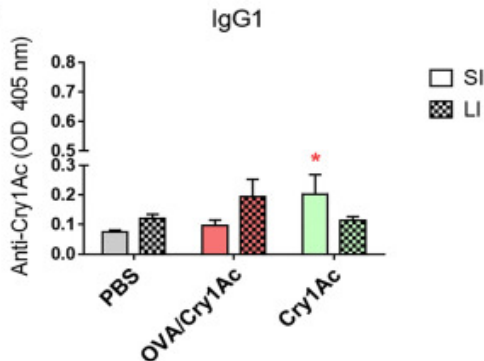
Binding of OVA-FITC to RAW264.7 macrophages is partially inhibited by preincubation with an excess of Cry1Ac. Competition assays were performed in RAW264.7 cells incubated at 4 °C in binding buffer as described in materials and methods. Cells were preincubated for 30 min on ice with 200 µg/mL of unlabeled Cry1Ac or OVA or BSA (as unrelated control protein) and then incubated for 30 min on ice with OVA-FITC (3 µg/mL). Cells were analyzed by flow cytometry; a representative histogram is shown. The result shown was obtained from two independent experiments performed in duplicate.



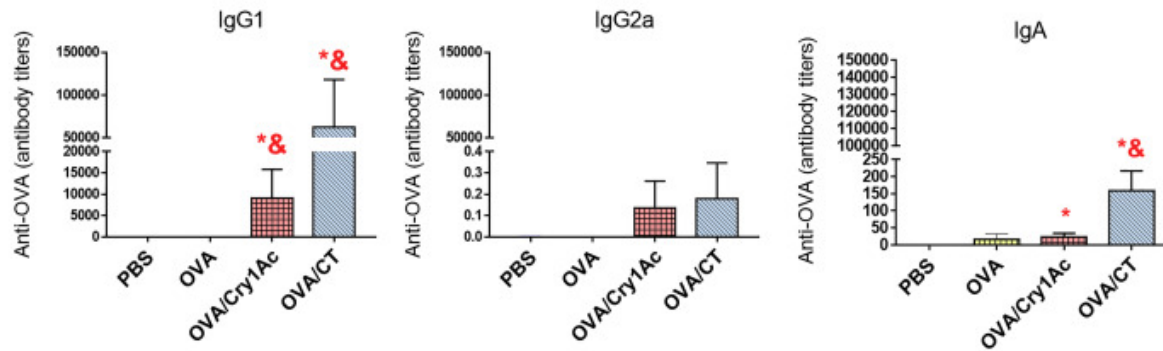
A



B



A



B

